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DETECTION OF THE AMPHIBIAN PATHOGEN *BATRACHOCHYTRIUM DENDROBATIDIS* ON SPADEFOOTS IN THE KAMLOOPS REGION

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DETECTION OF THE AMPHIBIAN PATHOGEN *BATRACHOCHYTRIUM DENDROBATIDIS* ON SPADEFOOTS IN THE KAMLOOPS REGION

by

KATIE BENNETT

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We accept this thesis as conforming to the required standards:

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ABSTRACT

Thesis Supervisor: Dr. Jonathan Van Hamme

Batrachochytrium dendrobatidis is a species of chytrid fungus responsible for the decline and extinction of amphibian populations worldwide (Longcore 1999 and others). Recently, *B. dendrobatidis* has been detected on western toads (*Bufo boreas*) in southwestern British Columbia (Deguise & Richardson 2009) and has therefore become a concern for conservationists across the province. Chytrid DNA can be extracted from tissue samples, skin swabs, and water bodies and subsequently detected using real time PCR techniques. It is currently unknown whether the chytrid fungus is affecting amphibian populations in the Kamloops area. This project aimed to first validate an effective real time PCR assay for detecting the presence of the amphibian pathogen *B. dendrobatidis*, and second to employ this real time PCR assay to detect *B. dendrobatidis* on experimental samples collected in the Kamloops region.

Specifically, spadefoots were captured during the summer of 2013 from the New Afton mining site located south of Kamloops, BC. A total of 78 spadefoots were captured, swabbed, and subsequently weighed, measured and released. Parameters for optimizing an effective real time PCR assay were tested to detect *B. dendrobatidis* in experimental samples; initially a Taqman assay was tried, but due to a lack of success, a SYBR Green assay was used. DNA was extracted from all samples and 30 of these samples were tested for *B. dendrobatidis* using the SYBR Green assay and gel electrophoresis.

B. dendrobatidis DNA was not detected in any of the 30 experimental samples. Melt curve analysis indicated the presence of multiple PCR products, products which were observed by gel electrophoresis at lengths less than 150 base pairs. It is possible that non-specific primer binding occurred, or that primer dimers formed, at low template DNA concentrations as these bands were not observed above 42 standard DNA copy numbers. Based on the results in hand, it is possible that (1) chytrid is not present in the ponds at New Afton mine, (2) chytrid is present but is not affecting the spadefoots, (3) the sensitivity of the SYBR Green assay was not sufficient to detect chytrid DNA, (4) PCR inhibitors interfered with the detection of chytrid DNA on samples, and (5) chytrid DNA did not adhere to the swabs that were used in this work. Future research should employ swabs with smaller tips

and employ the more sensitive Taqman assay using a probe with a non-flourescent quencher (NFQ) as opposed to a black-hole quencher (BHQ-1) that was used here.

ACKNOWLEDGEMENTS

This project would not have been possible without the help and contributions of Dr. Jon Van Hamme, Dr. Karl Larsen, and Joanne Hales. Jon was incredibly helpful with the molecular techniques used to test the environmental samples for chytrid and taught me many useful skills. He also spent a lot of time helping me to edit my honours proposal, U-REAP report, and honours thesis. Karl was always available when I had questions about spadefoots and played an integral role in getting this project started. His vast knowledge of spadefoots made summer data collection and capturing of the spadefoots much easier. During the summer, I was lucky enough to work alongside Joanne Hales, a Masters of Sciences student who is studying the ecology of spadefoots at New Afton. Joanne took me on as an undergraduate research assistant to help with her project but also helped me to collect data for my research.

In addition, this project was made more feasible by funding from New Afton, the Natural Sciences and Environmental Research Council of Canada, and the Undergraduate Research Experience Program (U-REAP) at Thompson Rivers University.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

1 INTRODUCTION

Batrachochytrium dendrobatidis is a species of chytrid fungus responsible for the decline and extinction of amphibian populations worldwide (Longcore 1999 and others). The fungus may be present in water bodies where it infects the keratinized cells of amphibians causing chytridomycosis; a disease that interrupts the ability of amphibians to osmoregulate (maintain proper water movement across skin) (Daszek *et al.* 1999 and others). Chytrid acts as a pathogen by disrupting electrolyte transport by inhibiting epithelial Na^+ channels; in severe cases, systemic depletion of Na^+, K^+, and Cl^- causes cardiac arrest (Campbell *et al.* 2011). *B. dendrobatidis* infects the skin of adult amphibians and the mouthparts of anuran larvae (Garner *et al.* 2005).

Currently, the chytrid fungus is associated with the rapid decline or extinction of approximately 200 amphibian species around the globe in an array of climates (Skerratt *et al.* 2007). The prevalence of *B. dendrobatidis* in an African population of *Xenopus* since the 1930's has been well documented. It is thought that international trade of infected *Xenopus laevis* by humans has acted as a vector for the transmission of chytrid to unaffected amphibian populations worldwide (Vredenburg *et al.* 2013). Presently, *B. dendrobatidis* is affecting amphibians on all continents; 52 of 82 countries sampled for the presence of the chytrid fungus have tested positive while the pathogen has been detected in 42 percent of amphibian species (Olson *et al.* 2013).

Recently, *B. dendrobatidis* has been detected on northern leopard frogs (Rana pipiens) in southeast British Columbia (Voordouw *et al.* 2010) and on western toads (*Bufo boreas*) in southwestern British Columbia (Deguise & Richardson 2009) and has therefore become a concern for conservationists across the province. This species of chytrid fungus can be detected in water bodies, sediments, and on amphibians through the use of real time PCR techniques (Boyle *et al.* 2004, Kirshtein *et al.* 2007). Two commonly used assays are the TaqMan assay and the SYBR Green assay. The TaqMan assay is frequently used (Boyle *et al.* 2004, Garner *et al.* 2006, and others) because it has a high specificity for the target DNA as it utilizes both a set of PCR primers as well as a nucleotide probe targeting a specific DNA sequence between the forward and reverse primer sites. The SYBR green assay has been

proven to be as equally sensitive as the TaqMan assay (Kirshtein*et al.* 2007), but is less common because it can have reduced specificity for the target DNA as it does not include the TaqMan probe, only the forward and reverse PCR primers. Chytrid may also be identified using histological techniques but this has proved less effective (Kriger *et al.* 2006).

Real time PCR is a highly efficient method for detecting *B. dendrobatidis* because it not only detects the presence or absence of the fungus in samples, but also provides information on the amount of fungus present in the original sample. It is currently unknown whether the chytrid fungus is affecting amphibian populations in the Kamloops area, and so this project aimed to firstly validate an effective real time PCR program that can be used to detect for the presence of the amphibian pathogen *B. dendrobatidis*, and secondly to employ this modified real time PCR program to determine whether this species of chytrid fungus is affecting Great Basin Spadefoots found on the New Afton mining site operated by New Gold Inc. located south of Kamloops, BC. New Afton's tailing ponds, as well as other ephemeral and permanent water bodies in the area, are commonly used as breeding ponds by spadefoots.

The Great Basin Spadefoot is an endangered amphibian found throughout the dry, arid region of western North America (Cannings 1999). There is very little known about the ecology of this species as it spends the majority of its life cycle in underground burrows. The ability to live in arid environments is unique to the spadefoots; their ability to absorb water from the soil (Ruibal *et al.* 1969) renders them capable of living underground for long periods of time. The spadefoots emerge from their burrows during breeding season; thunder and spring rains trigger the spadefoots to travel to breeding ponds (Ruibal *et al.* 1969). Breeding ponds may be ephemeral or permanent aquatic bodies including both large ponds present year-round and small puddles. Even minimal amounts of water in tire tracks left by vehicles can be sufficient for spadefoot breeding (Personal Observation). Tadpole development is rapid in order to increase their chances of survival, this is particularly important in ephemeral water bodies.

While some research studying *B. dendrobatidis* and amphibians has been undertaken in western North America (Deguise & Richardson 2009), no observations on the effects of *B. dendrobatidis* on the Great Basin Spadefoot have been made. Early detection of this parasitic fungus is crucial in helping to treat and protect endangered populations. In particular, the

knowledge of the relationship between *B. dendrobatidis* and the Great Basin Spadefoot can aid in development of a management plan to conserve this spadefoot species.

2 MATERIALS AND METHODS

2.1 Field Sampling

Collection of water and skin samples took place during the summer of 2013 on the New Afton mining site operated by New Gold Inc. Materials for collecting skin swabs included: sterile fine tip cotton swabs (Puritan Medical Products, Maine, part no. 25-806 1WC FDNA), alcohol, microcentrifuge tubes (Sarstedt, part no. 72.693.005), and sterile disposable, powder free vinyl gloves (Siedman Associates, New York, part no. GVP9-MD-1). Vinyl gloves were chosen as they have proven to have minimal toxicity for tadpoles relative to latex and nitrile gloves (Cashins *et al.* 2008). Each spadefoot was caught and handled using clean gloves. A cotton swab was repeatedly run over the specimen's abdomen, sides, drink patch, thighs, and feet webbing as outlined in Hyatt *et al.* (2007). The tip of the cotton swab was then clipped using scissors into a clean microcentrifuge tube and placed in a cooler on ice until it could be stored in a -20^{O}C freezer in the microbiology lab at Thompson Rivers University. Each spadefoot was weighed and its snout-vent length was measured to the nearest 0.5 mm using a measuring tape. The location and time of capture was also recorded.

2.2 Real Time PCR

This research took place in the microbiology lab at Thompson Rivers University during the fall of 2013 under the supervision of Dr. Jon Van Hamme. An Illumina Real Time PCR (rtPCR) instrument with Eco software was used for all rtPCR experiments. Materials ordered for this work included: *B. dendrobatidis* (*Bd*) rDNA control plasmids (Pisces Molecular, Colorado), Taqman Universal Master Mix II (Applied Biosystems, Ontario), SYBR Green buffer (Applied Biosystems, Ontario, part no. 4472908), a probe (Chytr MGB2), and forward and reverse PCR primers (ITS1-3 Chytr and 5.8S Chytr) (Alpha DNA, Quebec) (Figure 2.1). The primer and probe sequences chosen were taken from Boyle *et al.*

(2004) with the exception of a black hole quencher (BHQ-1) being attached to the probe instead of a non-fluorescent quencher (NFQ).

Primer Sequences

ITS1-3 Chytr 29 bases

5'- CCTTGATATAATACAGTGTGCCATATGTC -3'

5.8S Chytr 22 bases

5'- AGCCAAGAGATCCGTTGTCAAA -3'

Minor groove binder probe sequence

Chytr MGB2 15 bases

5'- 6FAM GCAGTCGAACAAAAT BHQ1 -3'

Figure 2.1 *Batrachochytrium dendrobatidis* primer sequences and minor groove binder probe sequence.

The 7 Bd control plasmid solutions ordered from Pisces Molecular ranged in concentration from 2.1 x 10^0 to 2.1 x 10^6 molecules/µL. Prior to rtPCR experiments, the control plasmids were each aliquoted into 10 microcentrifuge tubes and stored in a non-frost free freezer at -20°C in order to reduce the number of freeze-thaw cycles each standard was subjected to. Lyophilized PCR primers were briefly centrifuged and reconstituted in sterile, nuclease free water to a stock concentration of 100µM. Each stock was diluted to 900 nM and aliquoted into microcentrifuge tubes that were then stored at -20°C. The Chytr MGB2 probe was diluted with water to a final concentration of 250 nM and stored as well. Prior to each experiment, the primers, probe, and *Bd* plasmids were thawed on ice, vortexed and centrifuged. All equipment used for preparing PCR plates (e.g. pipettors, pipette tips) were placed under a UV light for approximately 2 minutes, and all reactions were prepared in a sterile biological safety cabinet using filter tips (Neptune Scientific, Ontario).

Following each rtPCR assay, PCR products were further analyzed using gel electrophoresis. The agarose gels used for *B. dendrobatidis* control plasmids were each prepared using 50 mL of Tris-borate EDTA (TBE) buffer and 0.75 g of agarose. Once the agarose was added to the TBE, the mixture was heated in a microwave until the agarose

dissolved (approximately 1 min 15 s). Next, 2.5 µL of GelRed (10 000 × Biotium, Lot 10G1026) was added to the mixture that was then allowed to cool prior to pouring into a gel tray (90 cm^3) containing an 8 or 15 well comb. Once the gel was set, the comb was removed and the gel was placed in an electrophoresis unit containing TBE. 2 µL loading dye (10× Fast digest buffer, Fermentas, Lithuania, Lot 00050741) was added to each PCR product, and 10 µL from each was pipetted into the agarose gel. Additionally, 5 µL of molecular weight marker (20mg/µL, Invitrogen, Ontario, part no. 15628-050) was added to a single lane. Electrophoresis was carried out for 60 min at 95 V.

2.3 TaqMan assays

For the first TaqMan experiment, an rtPCR assay was conducted following the methods outlined in Boyle *et al.*(2004) with the exception of using 15 µL reaction volumes as opposed to 25 µL reaction volumes. The 15 µL reactions contained 7.5 µL TaqMan buffer, 900 nM (0.54 µL) of each primer, 250 nM (0.75 µL) of probe, 5.47 µL of water, and 0.2 µL of a DNA control plasmid or water. The PCR amplification conditions were 2 min at 50^OC then10 min at 95^OC; this was followed by 20 s at 95^OC and 1 min at 60^OC for 50 cycles. In subsequent rtPCR assays using the TaqMan buffer, the annealing temperature was altered (Table 2.1) but all other amplification conditions remained the same. In addition to experimenting with an array of annealing temperatures, the probe and DNA volumes used were modified (Table 2.1).

Table 2.1 Volumes and concentrations of components used in the TaqMan real time PCR assays.

Primer volume (µL)	Primer concentration (nM)	Probe volume (µL)	Probe concentration (nM)	DNA volume (µL)	DNA concentration (molecules/rxn)	Annealing Temperature (°C)
0.54	900	0.75	250	0.2	$4.2 \times 10^{-1} – 4.2 \times 10^5$	60
0.54	900	0.75	250	0.2	$4.2 \times 10^{-1} – 4.2 \times 10^5$	58
0.54	900	0.75	250	0.2	$4.2 \times 10^{-1} – 4.2 \times 10^5$	62
0.54	900	0.75	250	0.2	$4.2 \times 10^{-1} – 4.2 \times 10^5$	64
0.54	900	0.75	250	0.2 – 2.2	$4.2 \times 10^5 – 4.62 \times 10^6$	66
0.54	900	0.75	250	0.2	$4.2 \times 10^{-1} – 4.2 \times 10^5$	60
0.54	900	0.75 – 2.5	250	0.2	4.2×10^5	60
0.54	900	0.75	250	0.2, 0.6, 0.8	4.2×10^5, 1.26×10^6, 1.68×10^6	59
0.54	900	2.5	250	0.2	$4.2 \times 10^{-1} – 4.2 \times 10^5$	59

0.54	900	2.5	250	0.2, 0.6, 0.8	4.2×10^5	59
0.54	900	2.5	250	0.2, 0.6, 0.8	4.2×10^5	57

2.4 SYBR Green assays

The first SYBR Green rtPCR experiment conducted used 15 µL reaction volumes. The 15 µL reaction volumes contained 7.5 µL SYBR Green buffer, 900 nM (0.54 µL) of each primer (ITS1-3 and 5.8S), 6.42 µL water, and 0.2 µL DNA control plasmid or water. The PCR amplification conditions were 2 min at 50°C then 2 min at 95°C; this was followed by 15 s at 95°C and 1 min at 59°C for 50 cycles. In addition, all but the first SYBR Green assay used a melt curve analysis of 15 s at 95°C, 15 s at 55°C, and 15 s at 95°C. Subsequent SYBR Green rtPCR experiments followed the same amplification conditions with the exception of the annealing temperature (Table 2.2). Finally, the last SYBR Green rtPCR experiment used four different primer concentrations and only the highest plasmid DNA concentration (4.2×10^5 molecules/reaction) (Table 2.2). This was done to explore an association between the primer concentrations used and the amount of primer dimer produced.

Table 2.2 Volumes and concentration of components used in the SYBR Green real time PCR assays.

Primer volume (µL)	**Primer concentrations (nM)**	**DNA concentration (molecules/rxn)**	**DNA volume (µL)**	**Annealing temperature (°C)**
0.54	900	$4.2 \times 10^{-1} – 4.2 \times 10^5$	0.2	60
0.54	900	$4.2 \times 10^{-1} – 4.2 \times 10^5$	0.2	60
0.54	900	$4.2 \times 10^{-1} – 4.2 \times 10^5$	0.2	60
0.54	900	$4.2 \times 10^{-1} – 4.2 \times 10^5$	0.2	60
0.54	900	$4.2 \times 10^{-1} – 4.2 \times 10^5$	0.2	62
0.54	900	$4.2 \times 10^{-1} – 4.2 \times 10^5$	0.2	66
0.54	900	$4.2 \times 10^{-1} – 4.2 \times 10^5$	0.2	68
0.3, 0.5, 0.7, 0.9	900	4.2×10^5	0.2	66

2.5 DNA extractions

DNA extractions were completed in the microbiology lab at Thompson Rivers University under the supervision of Dr. Jon Van Hamme during the winter of 2014. DNA extractions followed the methods outlined in Boyle *et al.* (2004) with the exception of 50 µL of PrepMan Ultra, rather than 40 µL, being used. In addition, Boyle *et al.* (2004) used whole toes to extract DNA from and we used cotton swabs as described by B.C. Ministry of Environment Wildlife Health Program (2008). Materials used for the extractions include: PrepMan Ultra and Zirconium/silica beads (0.5 mm in diameter). The cotton swabs used for sampling in the summer were quite large and so prior to nucleic acid extraction with PrepMan Ultra, fragments (approximately 0.001 g dry weight) were removed from the tip of each swab using a razor blade. This was carried out in a sterile biological safety cabinet using clean gloves. Between each swab, the razor blade and tweezers were placed in alcohol then flamed using a Bunsen burner. This helped to avoid cross-contamination of each sample. Each cotton sample was placed in a clean, labeled microcentrifuge tube containing 30 to 40 mg zirconium/silica beads. The samples were then stored in a non-frost free freezer at -20°C.

Fifty µL PrepMan Ultra was added to each prepared sample, the sample was then homogenized for 45 s in a mini beadbeater. This was followed by a brief centrifugation (30 s at $13 \times 10^3 \times g$) to recover all the material to the bottom of the tube. The homogenization and centrifugation steps were repeated once more prior to immersing the samples in a boiling water bath (approximately 102^{O}C) for ten minutes. After being cooled for 2 min in an ice water bath, the samples were centrifuged at $13\ 000 \times g$ for 3 min. Twenty µL of supernatent was recovered to a clean tube in a sterile biological safety cabinet and stored at -80°C.

2.6 Real Time PCR assays

Thirty environmental DNA samples were analyzed for *B. dendrobatidis* using a real time PCR SYBR Green assay. 15 µL reactions were prepared with each containing 7.5 µL SYBR Green buffer, 900 nM (0.54 µL) of each primer (ITS1-3 and 5.8S), 6.42 µL water, and 0.2 µL environmental DNA or *B. dendrobatidis* control plasmids. For each trial, environmental samples were run in triplicate and 2 standard curves were generated. The PCR

amplification conditions were 2 min at 50°C then 2 min at 95°C; this was followed by 15 s at 95°C and 1 min at 66°C for 50 cycles. In addition, a melt curve analysis consisting of a single cycle of 15 s at 95°C, 15 s at 55°C, and 15 s at 95°C was used.

To test for the presence of DNA inhibitors in environmental samples, a single rtPCR trial was run containing *B. dendrobatidis* plasmids alone as well as environmental samples spiked with plasmid DNA (Table 2.3). This experiment utilized both a SYBR Green buffer (as was used previously) and a SYBR Green Fastmix buffer (Quanta Biosciences, Gaithersburg, MD, part no. 95072-125) to compare the results of each buffer. The PCR amplifications used were 2 min at 50°C then 2 min at 95°C followed by 15 s at 95°C and 1 min at 66°C for 50 cycles.

Table 2.3 Experimental setup for a real time PCR assay using environmental samples spiked with *B. dendrobatidis* plasmids.

Lane	Column	Plasmid DNA (µL)	Plasmid DNA concentration (molecules/rxn)	Environmental DNA (µL)	Buffer
A	1	0.2	4.2×10^5	0	SYBR Green Fastmix
	2	0.2	4.2×10^4	0	SYBR Green Fastmix
	3	0.2	4.2×10^3	0	SYBR Green Fastmix
	4	0.2	4.2×10^2	0	SYBR Green Fastmix
	5	0.2	4.2×10^1	0	SYBR Green Fastmix
	6	0.2	4.2×10^0	0	SYBR Green Fastmix
	7	0.2	4.2×10^{-1}	0	SYBR Green Fastmix
	8	0	0	0	SYBR Green Fastmix
B	1	0.2	4.2×10^6	2.0	SYBR Green Fastmix
	2	0.2	4.2×10^6	1.0	SYBR Green Fastmix
	3	0.2	4.2×10^6	0.5	SYBR Green Fastmix
	4	0.2	4.2×10^6	0.25	SYBR Green Fastmix
	5	0.2	4.2×10^6	0	SYBR Green Fastmix
C	1	0.2	4.2×10^6	2.0	SYBR Green
	2	0.2	4.2×10^6	1.0	SYBR Green
	3	0.2	4.2×10^6	0.5	SYBR Green
	4	0.2	4.2×10^6	0.25	SYBR Green
	5	0.2	4.2×10^6	0	SYBR Green

2.7 Gel electrophoresis for experimental samples

Each of the 30 environmental DNA real time PCR products was analyzed using gel electrophoresis to confirm results of the rtPCR assay. Agarose gels were each prepared using 200 mL of tris-borate EDTA (TBE) and 3.0 g of agarose. Once the agarose was added to the TBE, the mixture was heated in a microwave until the agarose dissolved (approximately 1 min 50 s). Next, 10 µL of GelRed (Biotium, 10 000×) was added to the mixture that was then allowed to cool prior to pouring into a gel tray containing two 30 well combs. Once the gel was set, the comb was removed and the gel was placed in an electrophoresis unit containing TBE. Loading dye (2 µL of 10× Fast digest buffer, Fermentas) was added to each PCR product, and 5 µL from each was pipetted into the agarose gel. Additionally, 5 µL of molecular weight marker (20 mg/µL, Invitrogen, Ontario) was added to 4 lanes. Electrophoresis was carried out for 60 min at 95 V.

3 RESULTS

3.1 TaqMan assay

The TaqMan assay did not detect the Bd plasmid DNA and so a standard curve that could be used to detect fungal DNA in environmental samples was not produced. The first experiment followed the PCR program outlined in Boyle *et al.* (2004) and used DNA concentrations of 420 000, 42 000, 4 200, 420, 42, 4.2, and 0.42 molecules/reaction. Amplification plots (Figure 3.1) showed that no PCR products were produced and only background noise was detected. Following the real time PCR, agarose gel electrophoresis was used to visualize the quality of the PCR products. The agarose gel revealed unbound probe and primer dimer (Figure 3.2). The targeted 146 base pair region (Boyle *et al.* 2004) was not apparent. A possible reason for large amounts of unbound probe is the 60ºC annealing temperature was too high.

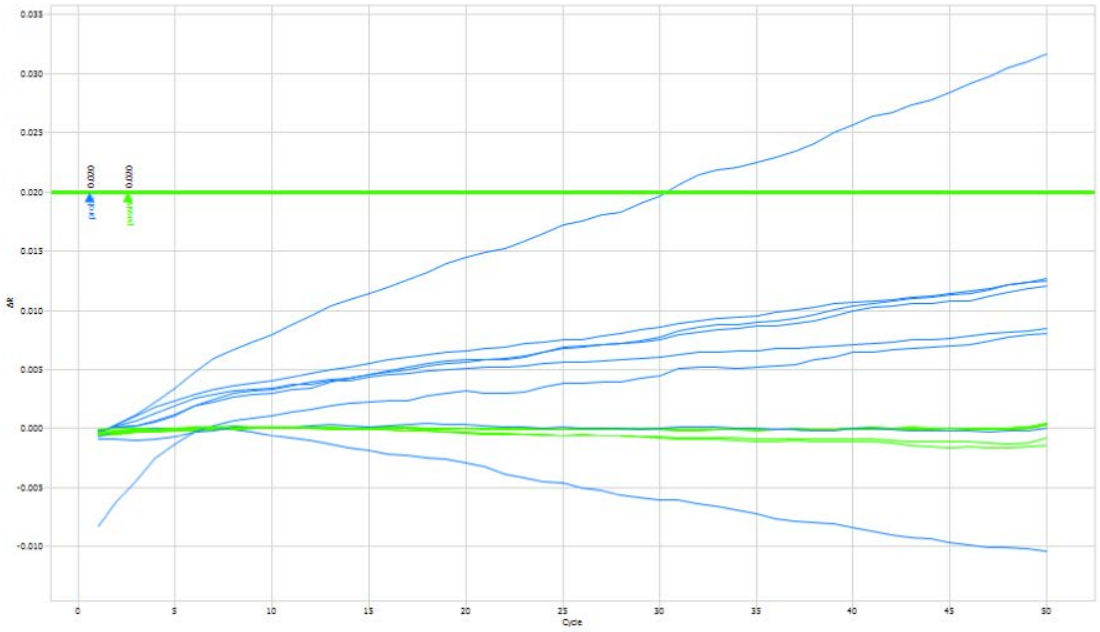

Figure 3.1 TaqMan assay amplification plot for chytrid standard DNA showing only background noise. Change in fluorescence is plotted against the cycle number.

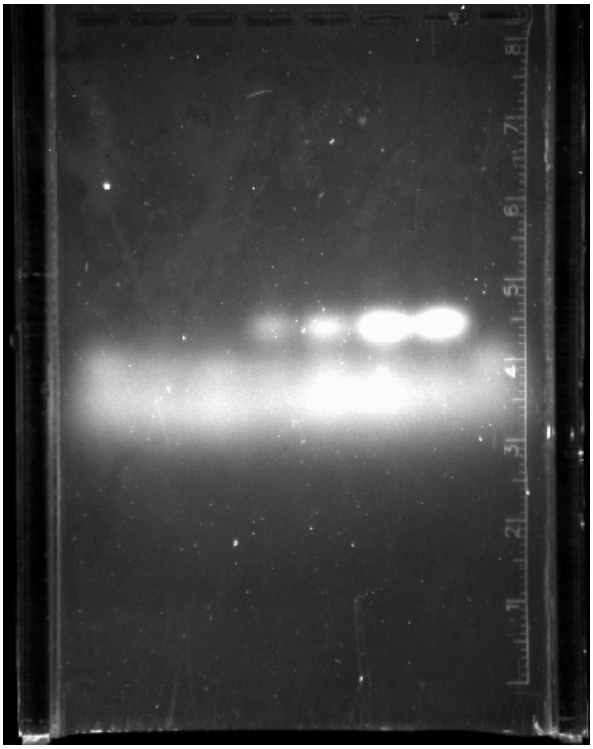

Figure 3.2 Agarose gel of real time PCR products from the first TaqMan assay. Lanes 1 through 8 contain DNA concentrations of 420 000, 42 000, 4 200, 420, 42, 4.2, 0.42 and 0 molecules/reaction respectively.

In the second TaqMan trial, a lower annealing temperature (58°C) was used to see if the probe would bind the targeted 146 base pair region more efficiently. All other parameters of the PCR program as well as the concentrations of the primers and probe plus DNA volumes remained the same as in the first experiment. The amplification plot (Figure 3.3) showed that no PCR products were produced and only background noise was detected. Gel electrophoresis was used to visualize the quality of the PCR products. As in the first experiment, a large amount of unbound probe was seen (Figure 3.4) and amplified *Bd* DNA was not apparent.

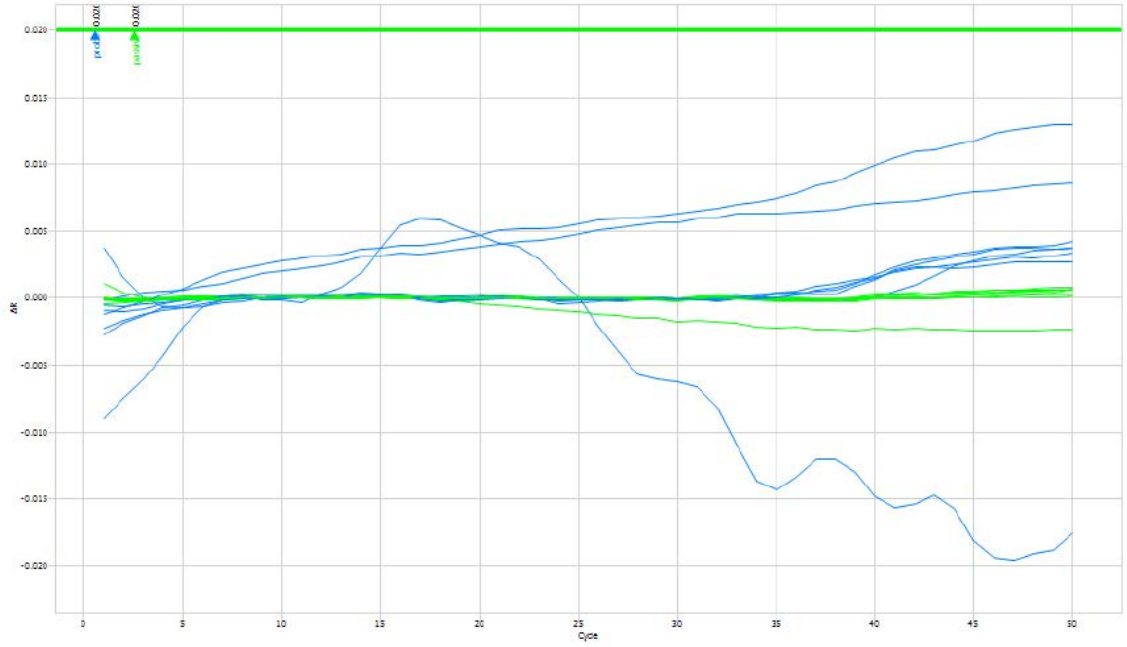

Figure 3.3 Second TaqMan assay amplification plot for chytrid standard DNA. Change in fluorescence is plotted against the cycle number.

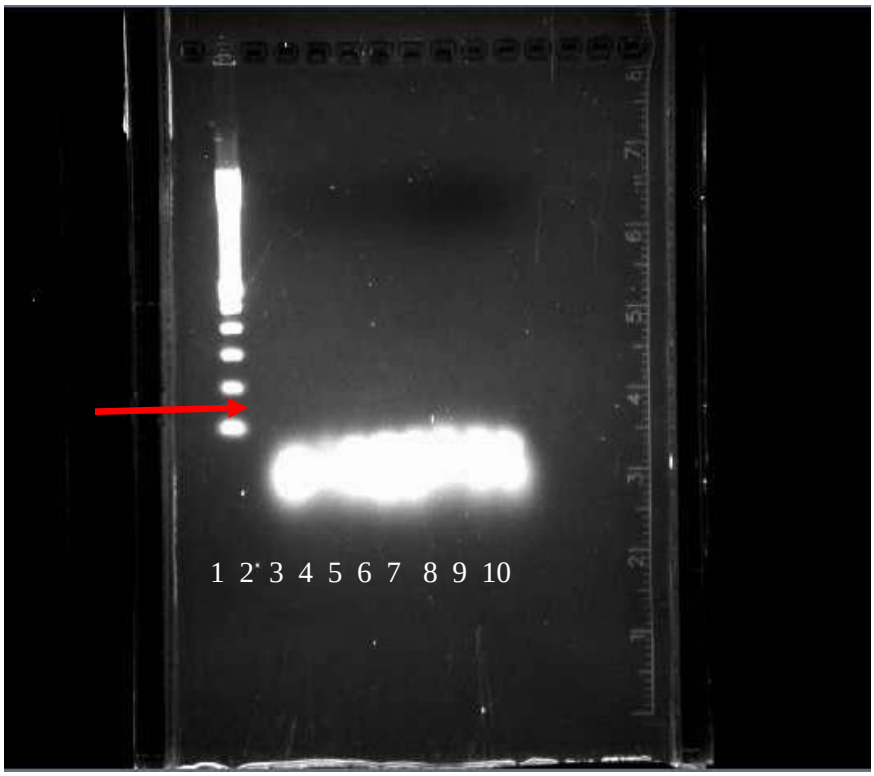

Figure 3.4 Agarose gel of real time PCR products from the second TaqMan assay. Lane 1 contains a molecular weight marker and lanes 3 through 10 contain DNA concentrations of 420 000, 42 000, 4 200, 420, 42, 4.2, .42, and 0 molecules/reaction respectively. Arrow indicates expected location of target DNA.

In the third TaqMan assay, a higher annealing temperature (62°C) was used to see if this enabled the probe and primers to bind the targeted region of DNA. All other parameters of the PCR program as well as the concentrations of the primers and probe plus DNA concentrations remained the same. The amplification plot (Figure 3.5) showed that minor PCR product was produced in one reaction but in all other reactions only background noise was detected, as in the previous two assays. This experiment was then repeated using an annealing temperature of 66°C, however, this assay yielded similar results (Figure 3.6).

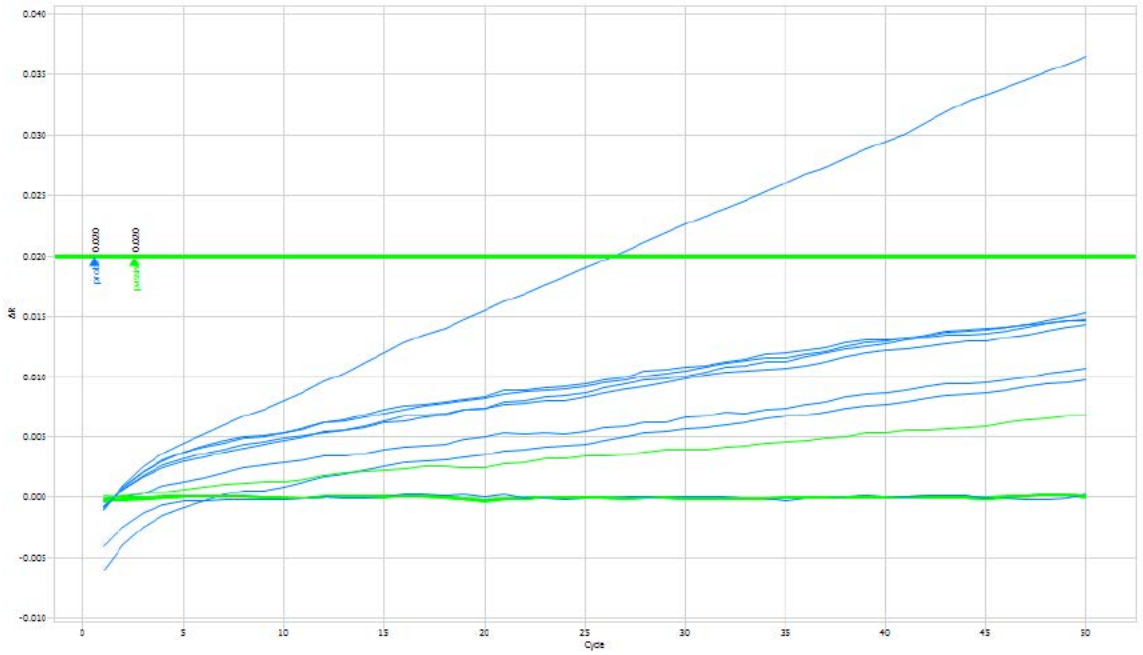

Figure 3.5 Third TaqMan assay amplification plot for chytrid standard DNA. Change in fluorescence is plotted against the cycle number.

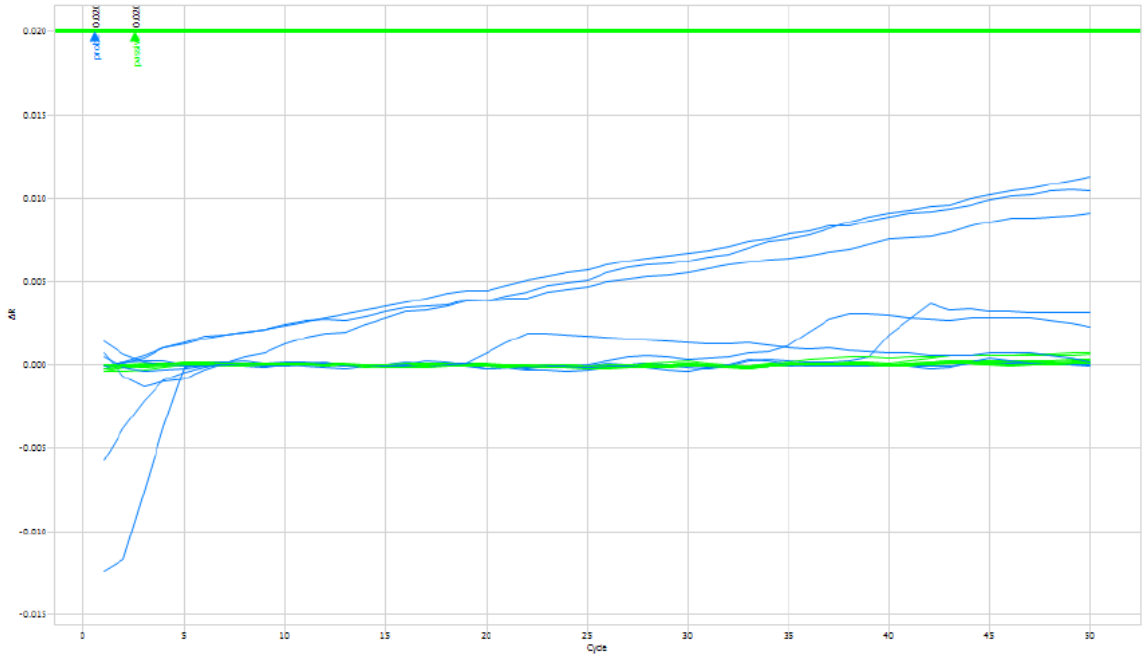

Figure 3.6 Fourth TaqMan assay amplification plot for chytrid standard DNA. Change in fluorescence is plotted against the cycle number.

Overall, annealing temperatures between 58°C and 62°C were tested but did not improve the results. Greater concentrations of DNA were also tested (4.2×10^5, 8.4×10^5, 1.26×10^6, 1.68×10^6, 2.1×10^6, 2.52×10^6, 2.94×10^6, 3.36×10^6, 3.78×10^6, 4.2×10^6, and 4.62×10^6 molecules/reaction) such as in the fifth TaqMan rtPCR assay. But, again, the target PCR product was not generated as shown in both amplification plots (Figure 3.7) and gel electrophoresis imaging.

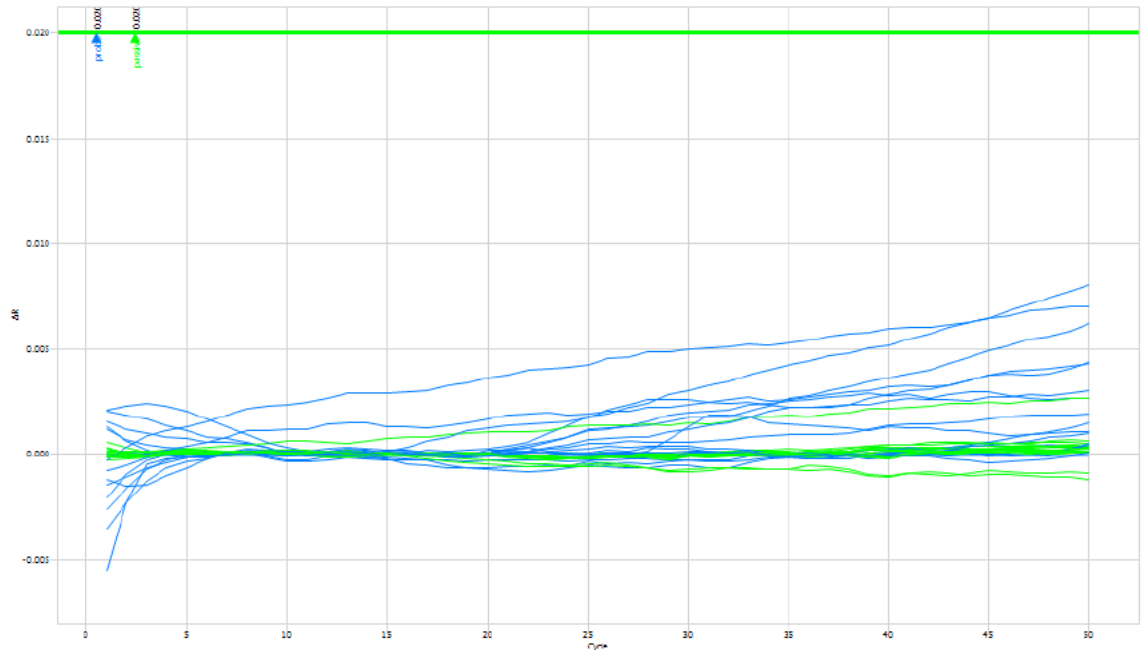

Figure 3.7 A typical TaqMan assay amplification plot for chytrid standard DNA. Change in fluorescence is plotted against the cycle number.

Given the lack of success, and the cost of the MGB-labelled probe, a SYBR green assay was tried. Other amplification plots and gels produced from the experimental TaqMan assays can be found in appendix A.

3.2 SYBR Green assays

Since it appeared that some PCR product was being generated in the latter TaqMan assays, but no fluorescent signal was being detected, a SYBR Green assay was tested using the PCR program described in Boyle *et al.* 2004 with DNA concentrations of 0.42, 4.2, 42,

420, 4 200, 42 000, and 420 000 molecules/reaction. PCR product was detected by rtPCR as shown in the amplification plot (Figure 3.8). Gel electrophoresis was used to visualize the quality of the PCR products; a large amount of product was seen (Figure 3.9) but there was also non-specific binding and primer dimer. In the following three experiments, this PCR program was repeated and the same concentrations of DNA, primer, and buffer were used to see if the results from the first trial would persist. In addition, a melt curve analysis was added to help determine the quality of the PCR products. All experiments yielded similar results.

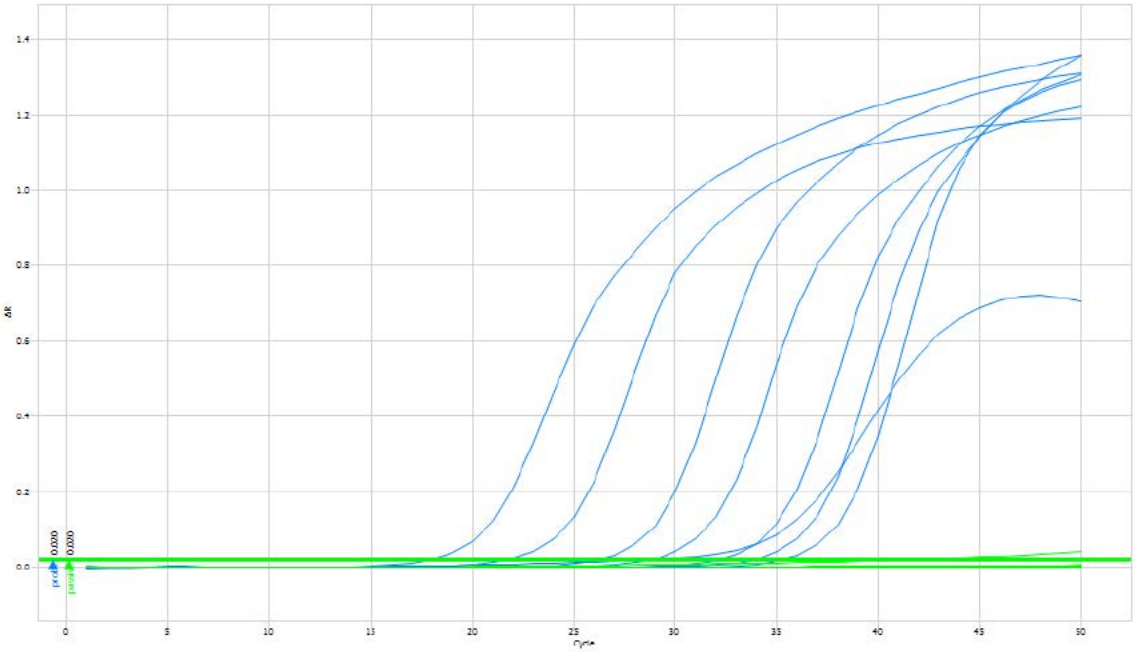

Figure 3.8 A SYBR Green assay amplification plot for chytrid standard DNA. Change in fluorescence is plotted against the cycle number.

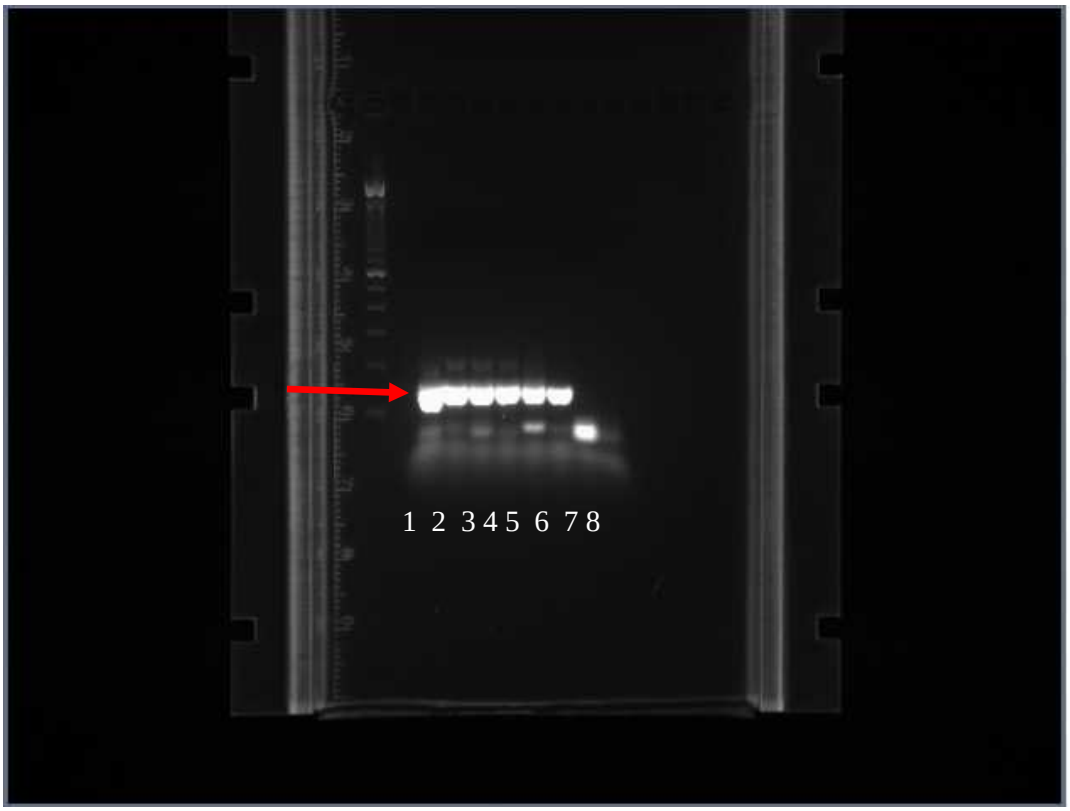

Figure 3.9 Agarose gel of real time PCR products from the first SYBR Green assay. Lanes 1 through 8 contain DNA concentrations of 420 000, 42 000, 4 200, 420, 42, 4.2, .42, and 0 molecules/reaction, respectively. Arrow indicates location of target DNA (146 bp).

In subsequent experiments, annealing temperatures of 62°C, 66°C, and 68°C were used to find an ideal temperature that would limit the amount of nonspecific binding and primer dimer formation. The optimal real time PCR conditions were found to be 50°C for 2 min then 95°C for 2 min followed by 50 cycles of 95°C for 2 min and 66°C for 1 min, followed by a melt curve analysis. The 15 µL reactions yielded the best results when they contained 7.5 µL SYBR Green buffer, 900 nM (0.54 µL) of each primer (ITS1-3 Chytr and 5.8S Chytr), 6.22 µL of water, and 0.2 µL of standard DNA.

The detection limit of this real time PCR program is 42 copies (Figure 3.10). Positive amplification above the threshold (C_T) after 35 cycles was not considered positive as non-specific PCR product was repeatedly detected in control wells (Figure 3.11). DNA concentrations at and above 42 molecules/reaction resulted in strong fluorescent signals before 35 cycles, excellent melt curves, and little or no non-specific PCR product (Figure

3.10, Figure 3.11, Figure 3.12, Figure 3.13). Points on a standard curve graph produced by fluorescent signals after 35 cycles resulted in poor R^2 values indicative of an inefficient PCR program. The melt curve analysis (Figure 3.12) revealed that the 5 reactions containing the greatest concentrations of DNA (420 000, 42 000, 4 200, 420, and 42 molecules/reaction) had PCR product melting at the same temperature (approximately 73^{O}C). This coincides with the gel images produced from the PCR products. In lanes 1 through 4 there is a large amount of amplified DNA with a length close to 146 base pairs, indicating this is the target DNA. In lanes 5 through 7 there is some PCR product but also a large amount of nonspecific product and in lane 8 there is no PCR product but lots of nonspecific product.

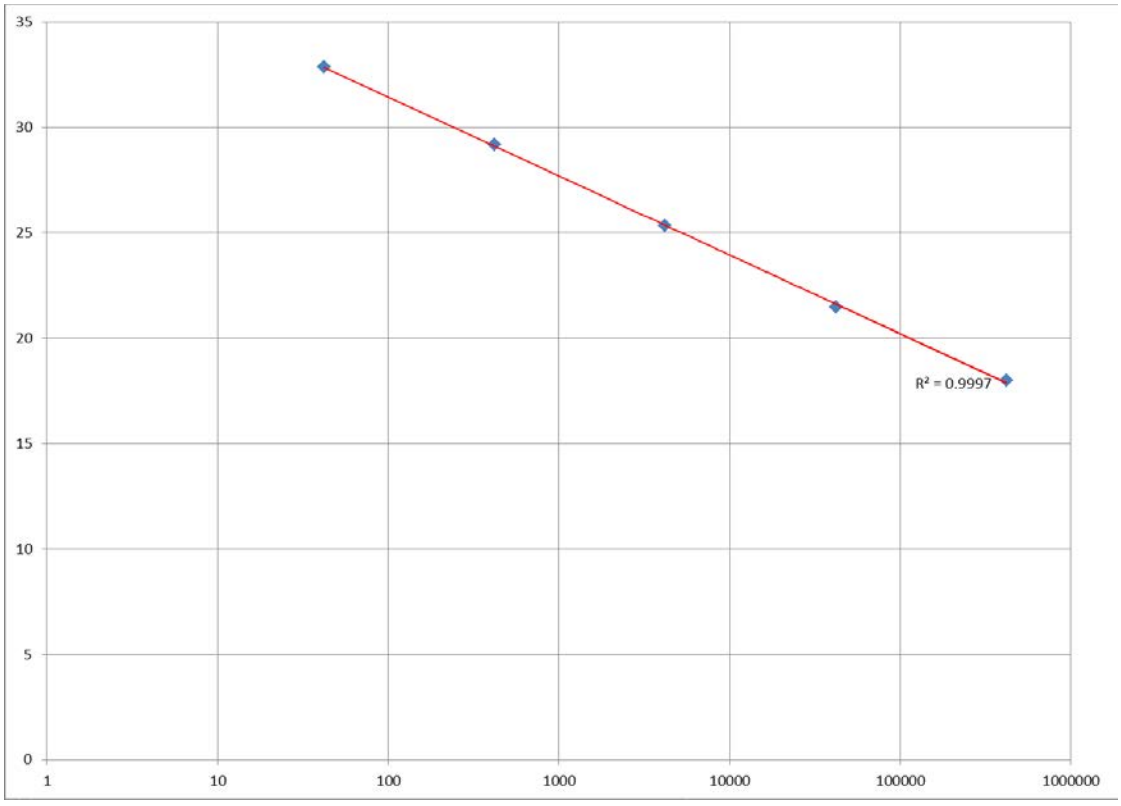

Figure 3.10 Standard curve generated from a series of dilutions of template DNA using the SYBR green buffer and a cycling temperature of 66°C. Copy number is on the x-axis and C_Q value is on the y-axis.

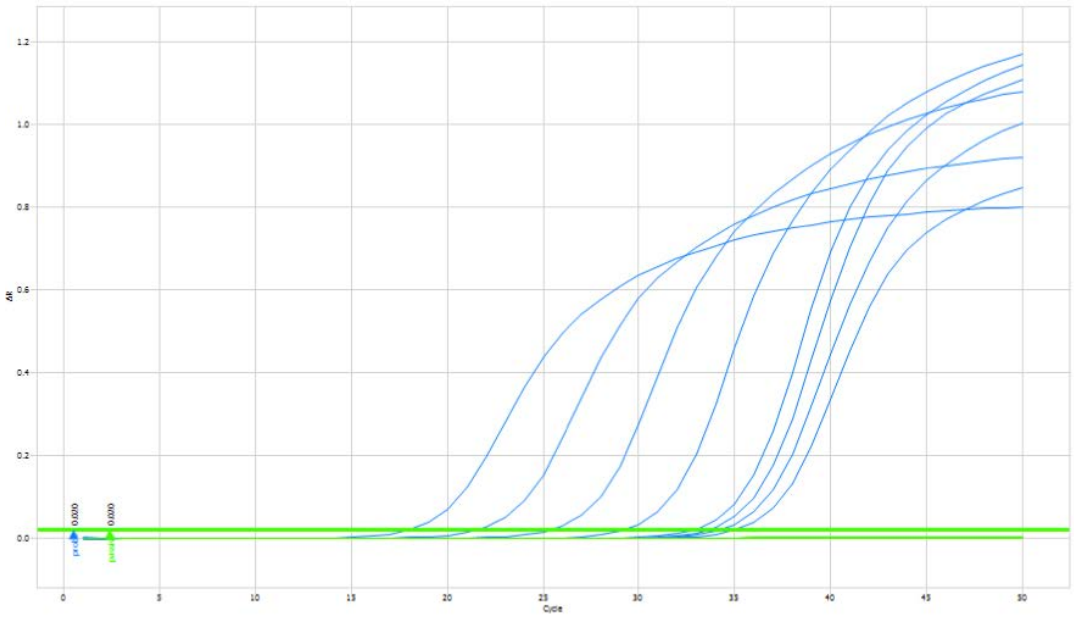

Figure 3.11 Amplification plot produced from a rtPCR SYBR green assay using standard DNA and a cycling temperature of 66°C.

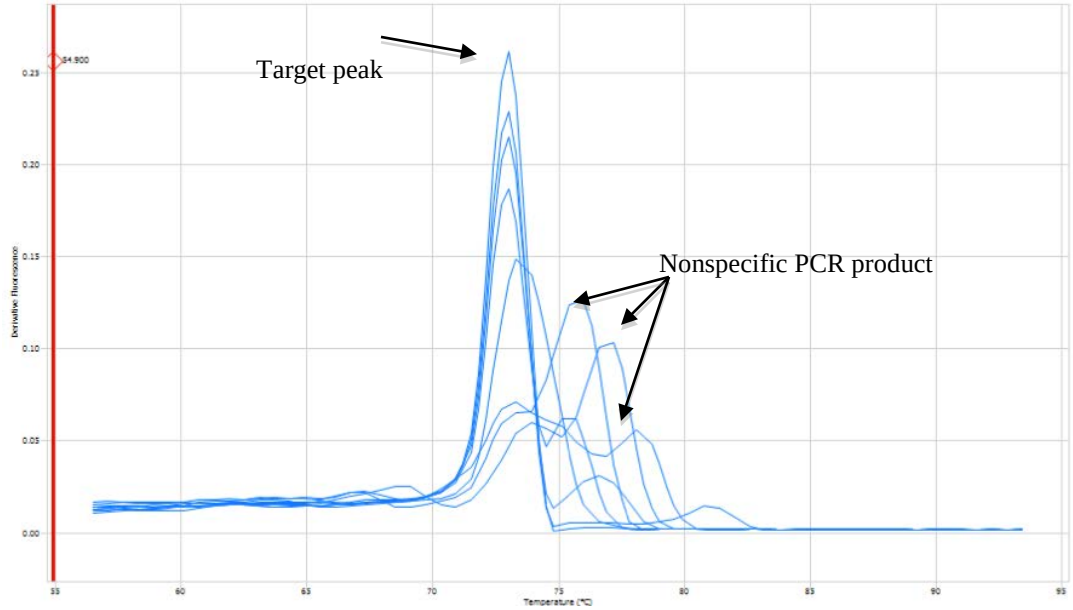

Figure 3.12 Melt curve analysis of a real time PCR trial using the SYBR green buffer run at a cycling temperature of 66˚C. Target DNA is located at approximately 73˚C.

Gel images produced from the real time PCR products helped to confirm the detection limit of the PCR program. The use of gel electrophoresis also revealed the formation of primer dimer and nonspecific binding (Figure 3.13).

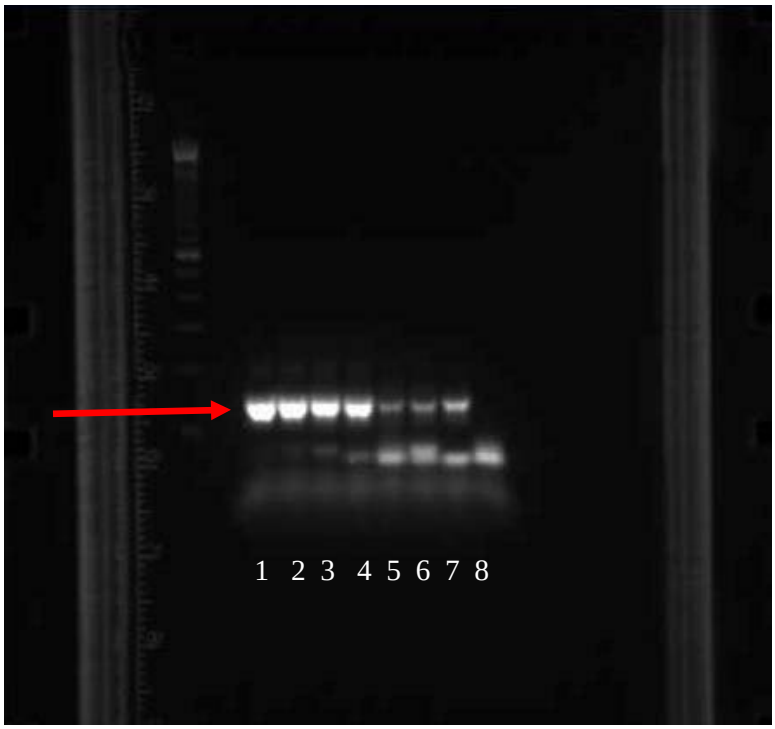

Figure 3.13 Gel electrophoresis produced using real time PCR products yielded from a cycling temperature of 66^{O}C. Lanes 1 through 8 contain DNA concentrations of 420 000, 42 000, 4 200, 420, 42, 4.2, .42, and 0 molecules/reaction respectively. Arrow indicates location of target DNA (146 bp).

3.3 Real Time PCR analysis of environmental samples

The real time PCR SYBR Green assay detected DNA in all experimental samples as well as wells containing standard DNA at concentrations of 420 000, 42 000, 4 200, 420, 42, 4.2, and 0.42 molecules/reaction (Figure 3.14). Though it appears as though there is chytrid in environmental samples, melt curve analyses (Figure 3.15) revealed the amplification of non-chytrid DNA in both control and experimental wells. The melt curve analysis for each rtPCR trial revealed that the 5 reactions containing the greatest concentrations of chytrid standard DNA (420 000, 42 000, 4 200, 420, and 42 molecules/reaction) had PCR product melting at the same temperature (approximately 73°C), however, all other products melted at

a greater temperature (approximately 78.5°C). This was consistent with the standard curve generated (Figure 3.16).

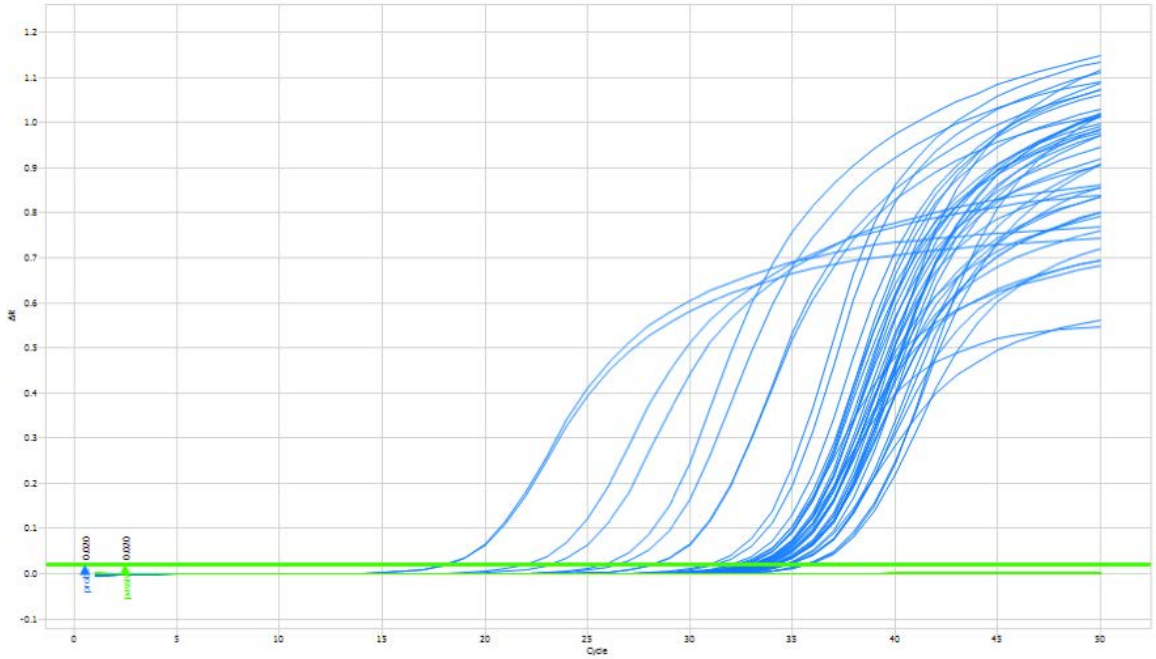

Figure 3.14 Amplification plot produced from a real time PCR SYBR green assay using both standard DNA and experimental DNA.

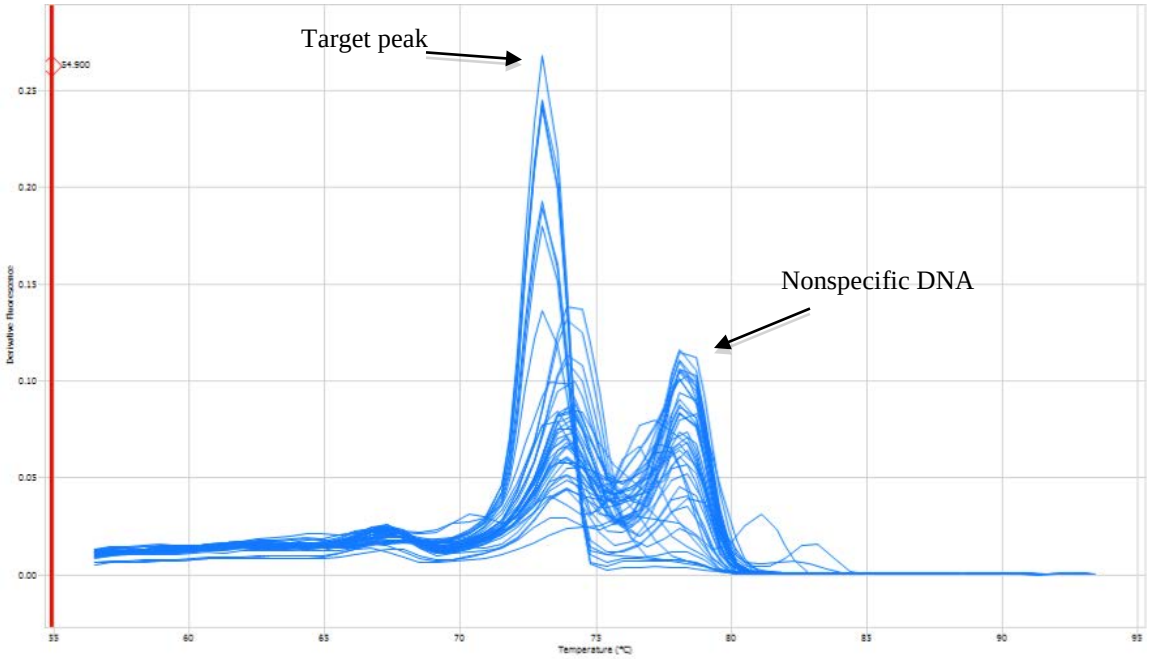

Figure 3.15 Melt curve analysis of a real time PCR trial using a SYBR Green buffer with both experimental samples and chytrid standard DNA.

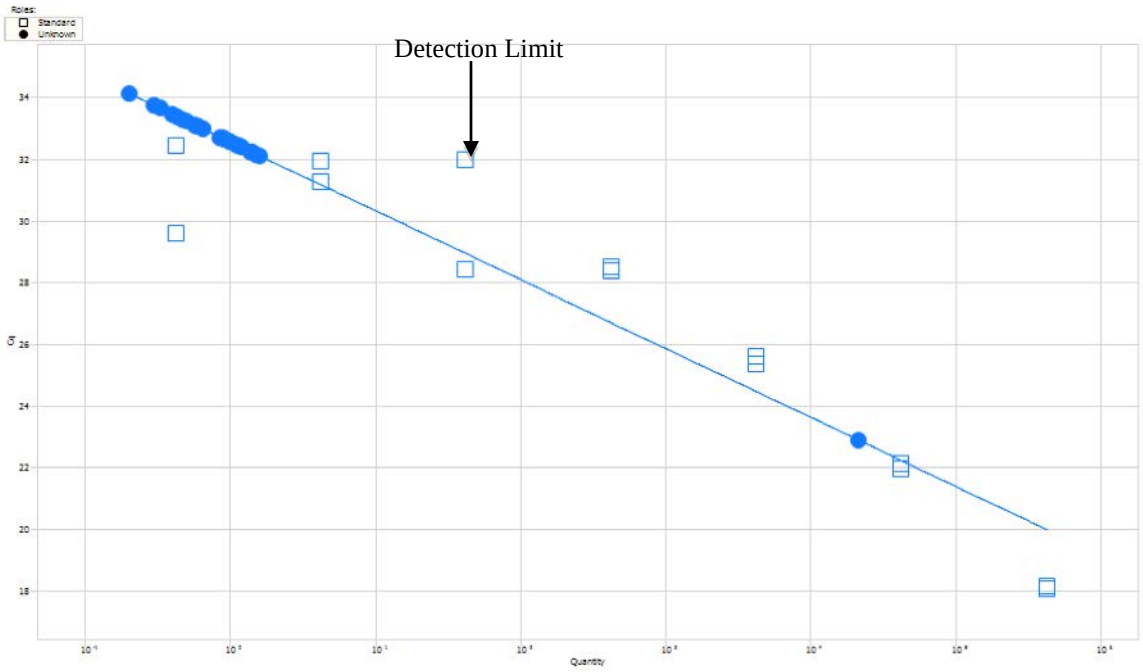

Figure 3.16 Standard curve plot produced from a real time PCR SYBR Green assay using experimental DNA and chytrid standard DNA at concentrations of 420 000, 42 000, 4 200, 420, 42, and 0.42 molecules/reaction. Open squares indicate standard DNA and solid circles indicate environmental samples.

Gel electrophoresis confirmed results of the melt curve analysis and rtPCR standard curve. Product was present in the wells containing standard DNA at concentrations of 420 000, 42 000, 4 200, 420, and sometimes 42 molecules/reaction (Figure 3.17) Thick bands below the targeted DNA sequence are likely non-specific PCR product. Other examples of gel images are in Appendix 1.

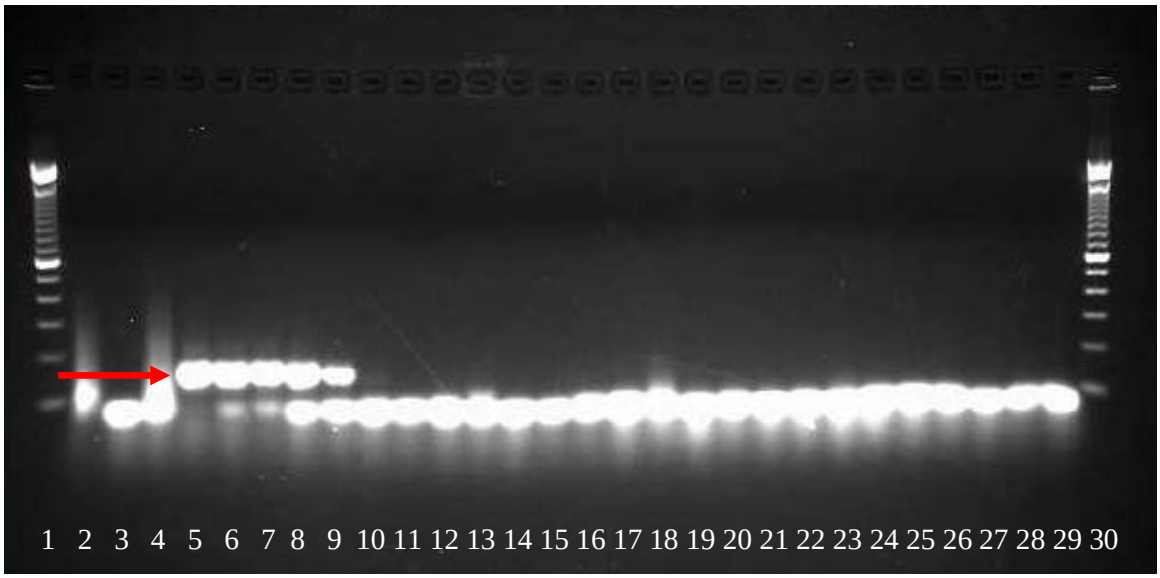

Figure 3.17 Gel electrophoresis produced using real time PCR products. Lanes 1 and 30 contain a molecular weight marker, lanes 2-4 and 13-29 contain environmental samples, and lanes 5 through 12 contain DNA concentrations of 420 000, 42 000, 4 200, 420, 42, 4.2, .42, and 0 molecules/reaction respectively. Arrow indicates location of target DNA (146 bp).

3.4 PCR Inhibitors

Spiking different volumes of environmental DNA samples with *B. dendrobatidis* standards revealed the presence of PCR inhibitors in the DNA extracted from swabs. For both the SYBR Green FastMix buffer and the regular SYBR Green buffer, samples containing smaller volumes of environmental DNA had earlier amplification cycles while those with greater volumes of environmental DNA either had later amplification cycles or did not have any amplification (Table 3.1). As the volume of environmental DNA in the samples decreased, the amplification cycles occurred earlier indicating the presence of more DNA in those wells.

Table 3.1 Amplification Cycle for environmental samples spiked with *B. dendrobatidis* plasmids using a real time PCR SYBR Green assay.

Lane	Column	Plasmid DNA (µL)	Plasmid DNA concentration (molecules/rxn)	Environmental DNA (µL)	Amplification Cycle
A	1	0.2	4.2×10^5	0	17
	2	0.2	4.2×10^4	0	22

	3	0.2	4.2×10^{3}	0	25
	4	0.2	4.2×10^{2}	0	26
	5	0.2	4.2×10^{1}	0	26
	6	0.2	4.2×10^{0}	0	26
	7	0.2	4.2×10^{-1}	0	26
	8	0	0	0	27
B	1	0.2	4.2×10^{6}	2.0	No amplification
	2	0.2	4.2×10^{6}	1.0	No amplification
	3	0.2	4.2×10^{6}	0.5	No amplification
	4	0.2	4.2×10^{6}	0.25	17
	5	0.2	4.2×10^{6}	0	17
C	1	0.2	4.2×10^{6}	2.0	43
	2	0.2	4.2×10^{6}	1.0	27
	3	0.2	4.2×10^{6}	0.5	20
	4	0.2	4.2×10^{6}	0.25	18
	5	0.2	4.2×10^{6}	0	17

In addition, the regular SYBR Green buffer proved more effective at detecting standard DNA than the SYBR Green FastMix buffer. The regular SYBR Green buffer detected product in all wells while the SYBR Green FastMix buffer only detected DNA in 2 wells.

4 DISCUSSION

Batrachochytrium dendrobatidis has negative implications for amphibian species around the globe as it infects keratinized skin cells causing chytridiomycosis; a condition that ultimately leads to mortality (Daszek *et al.*1999 and others). Due to the detection of chytrid on all continents (Olsen *et al.* 2013), many amphibian species are at risk of declining population sizes and even extinction. *B. dendrobatidis* can be detected using both histological and molecular techniques, however, molecular techniques have proven to be more effective (Kriger *et al.* 2006).

Real time PCR techniques using either a TaqMan or SYBR green assay are most commonly used to detect *B. dendrobatidis*. The rtPCR assay employed by Boyle *et al.* 2004

is often referenced. In their work, they compared the sensitivity and specificity of a TaqMan assay to histological detection methods, concluding that the TaqMan assay is more accurate than histology for detecting chytrid. Boyle *et al.* consistently generated standard curves with an R^2 value close to one (indicative of an efficient assay) and a detection limit of 0.1 genome equivalents. This is similar to the 10 copy detection limit for the SYBR Green and TaqMan assays employed by Kirshtein *et al.* 2007, but is lower than our detection limit of 42 copies. Kirshtein *et al*. experimented with a SYBR Green assay following the PCR program outlined in Boyle *et al.* 2004 and used melt curve analysis and gel electrophoresis to ensure PCR amplification did not result in the formation of primer dimer. This is the only study I found that mentioned the use of gel electrophoresis or melt curve analysis to ensure efficacy of the PCR program. No studies were found that included gel images or melt curve analysis descriptions in their results.

Real time PCR can be used to detect *B. dendrobatidis* in water bodies (Hyman & Collins 2012, Kirshtein *et al.* 2007), on amphibian skin swabs (Forzan *et al.* 2010, Hyman & Collins 2012, Kriger *et al*. 2006), and on amphibian toe clips (Deguise & Richardson 2006, Garner *et al.* 2006). In the present study, we chose to use skin swabs as opposed to toe clips because collecting skin swabs is considerably less invasive than toe clipping. In addition, toe clips may not provide sufficient sample for detecting the fungus (Boyle *et al.* 2004), while swab-PCR techniques have proven to be highly sensitive (Forzan *et al.* 2010, Hyman & Collins 2012, Kriger *et al*. 2006). In the literature, researchers have used wooden toothpicks (Hyman & Collins 2012) and rayon swabs (Kriger *et al.* 2006) to collect swab samples from animals. However, due to a limited budget, we opted to use a relatively large cotton swab. This posed difficulties as the cotton soaked up very large volumes of PrepMan Ultra, thus diluting the concentration of experimental DNA. To avoid the use of such large volumes of PrepMan Ultra, we shaved small amounts of cotton off the swab and extracted DNA from these pieces. In the future, it would be beneficial to use a rayon swab (Medical Wire & Equipment, MW 100-100).

Given previous experiments that detected chytrid using rtPCR, and through extensive trial and error, we successfully validated an effective real time PCR program that can be used to detect for the presence of the amphibian pathogen *B. dendrobatidis.* While the TaqMan assay yielded no results, the SYBR green assay was effective as it detected the control *B.*

dendrobatidis DNA and a good standard curve could be generated from the data. Note that the quencher used on the probe in the TaqMan experiments was a black hole quencher (BHQ), while the probe used in the literature was a non-fluorescent quencher (NFQ) (Boyle *et al.* 2004). This may have been a factor in the lack of results produced in the TaqMan assay, although we have no evidence to support this. The PCR program outlined in Boyle *et al.* 2004 was used with the exception of a 66˚C cycling temperature instead of a 60˚C cycling temperature.

Once the SYBR Green assay and gel electrophoresis methods were validated, they was used to identify the presence of the chytrid fungus on Great Basin Spadefoots found on the New Afton mining site located south of Kamloops, BC. Of the 30 environmental samples analyzed, none appeared to contain chytrid DNA. Possible explanations for the absence of chytrid are: (1) chytrid is not present in the ponds at New Afton mine, (2) chytrid is present but is not affecting the spadefoots, (3) the sensitivity of the SYBR Green assay was not enough to detect chytrid DNA that was potentially on the swabs, (4) PCR inhibitors prevented chytrid from being detected in the rtPCR assays, and (5) chytrid DNA did not adhere to the swabs.

Chytrid may not be present in the ponds at New Afton mine due to the ephemeral nature of many of the ponds. *B. dendrobatidis* requires a vector to establish itself in water bodies and so if the ponds are only present for short periods of time, it is possible that chytrid does not have enough time to grow in the ponds and in turn affect amphibian hosts. Alternately, chytrid may be present in the ponds at New Gold (particularly the permanent water bodies) but is not affecting the spadefoots. Spadefoots employ the ponds as breeding sites for only short periods each year; this may protect them from becoming infected as they have less contact with the water and pathogens within it (i.e. *B. dendrobatidis*) than other amphibians. A third explanation for chytrid not being detected on the environmental swabs is the sensitivity of the SYBR Green assay was not enough to detect chytrid DNA on the swabs. The unknown DNA present on environmental swabs was minimal (Appendix D) and the SYBR Green assay was only able to detect as little as 42 copies. Chytrid may also have not been detected due to polymerase inhibitors interfering with the copying of DNA. Polymerase inhibitors are detrimental to PCR results because they produce false negatives. The mechanism of action of PCR inhibitors varies; while some interfere with the annealing ability

of PCR primers, others act by degrading PCR polymerases (Schrader *et al.* 2012). PCR inhibitors may have originated in the field when the samples were collected, or in the laboratory during DNA extraction and processing. Finally, chytrid DNA may not have adhered to the swabs because we used swabs made of a cotton material as opposed to swabs made of rayon as used in the literature.

Future research should use a different type of swab (Medical Wire & Equipment, MW 100-100) and employ the more sensitive Taqman assay using a probe with a non-flourescent quencher (NFQ) as opposed to a black-hole quencher (BHQ-1). In addition, taking water samples and skin swabs from spadefoot anuran larvae and other amphibian species throughout the spring, summer, and fall could help to gain further insight into the association between chytrid and spadefoots in the Kamloops region. The samples collected could be tested for *B. dendrobatidis* using real time PCR techniques and gel electrophoresis.

Research conducted by Deguise and Richardson in 2009 revealed the presence of the chytrid fungus in a breeding population of Western Toads (*Anaxyrus boreas*) near Squamish, British Columbia. Given the close proximity of the study site with Kamloops, it is possible that chytrid may already be affecting amphibian populations near Kamloops or will potentially affect them in the future. Future research in the Kamloops region would improve our knowledge of the relationship between *B. dendrobatidis* and spadefoots as well as other amphibians in the area. This information could therefore aid in the development of a management plan to conserve amphibian species affected by the chytrid fungus. Possible management could involve the creation of man-made ponds containing chytrid-free water for spadefoot breeding or building toad fences around ponds containing chytrid.

5 LITERATURE CITED

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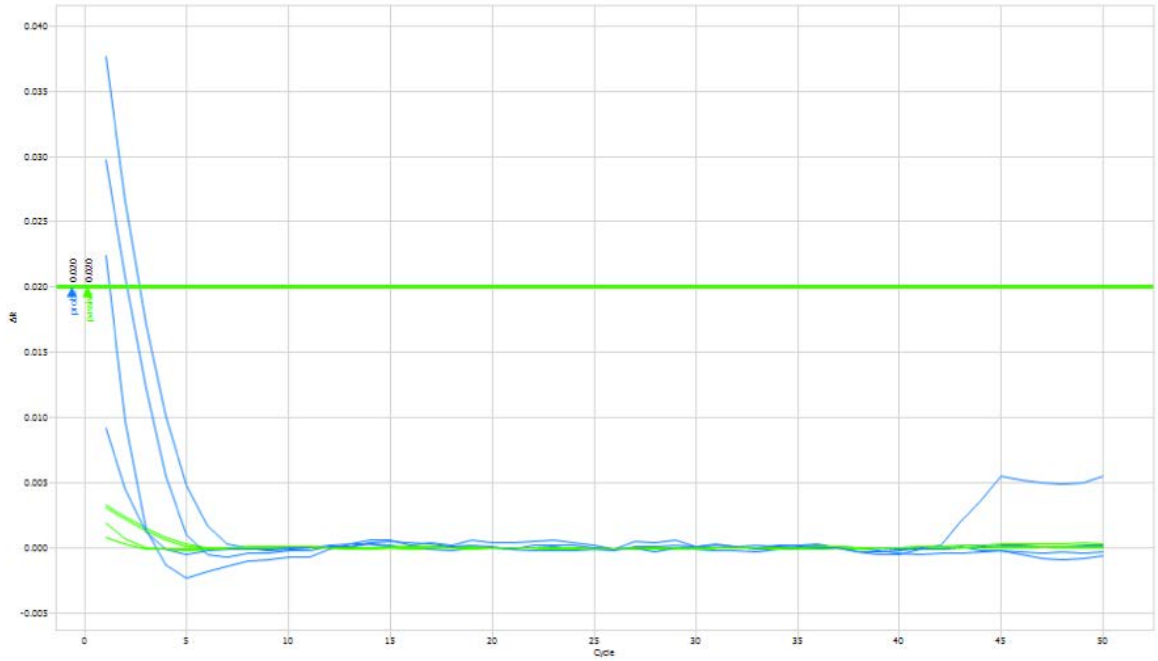
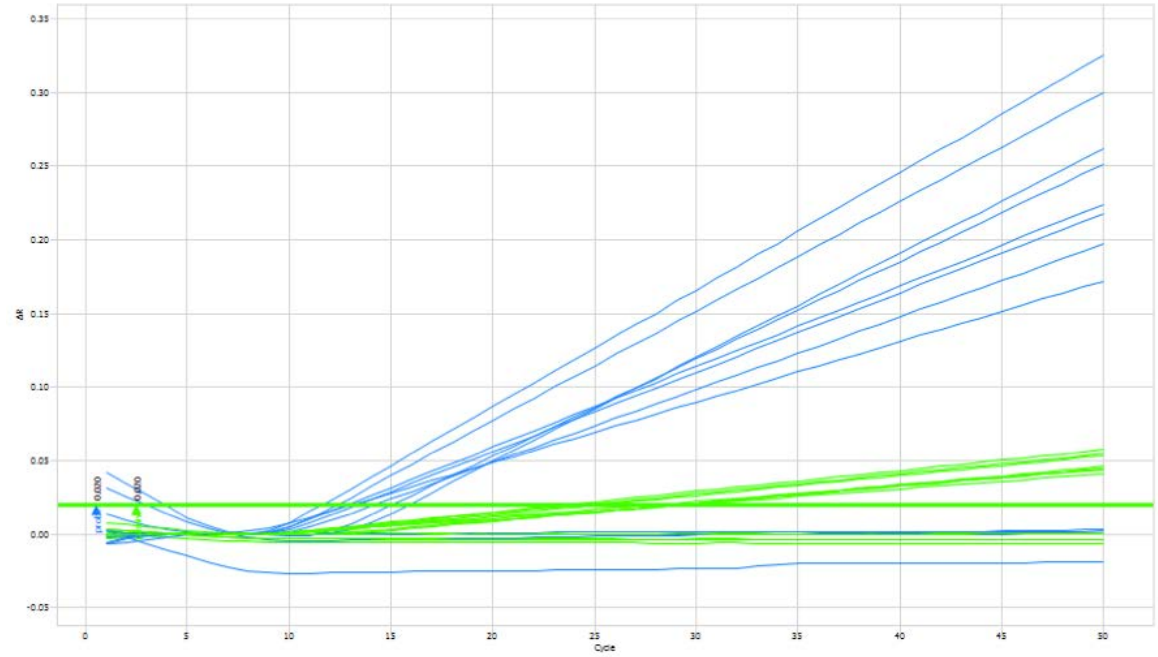
6 APPENDICES

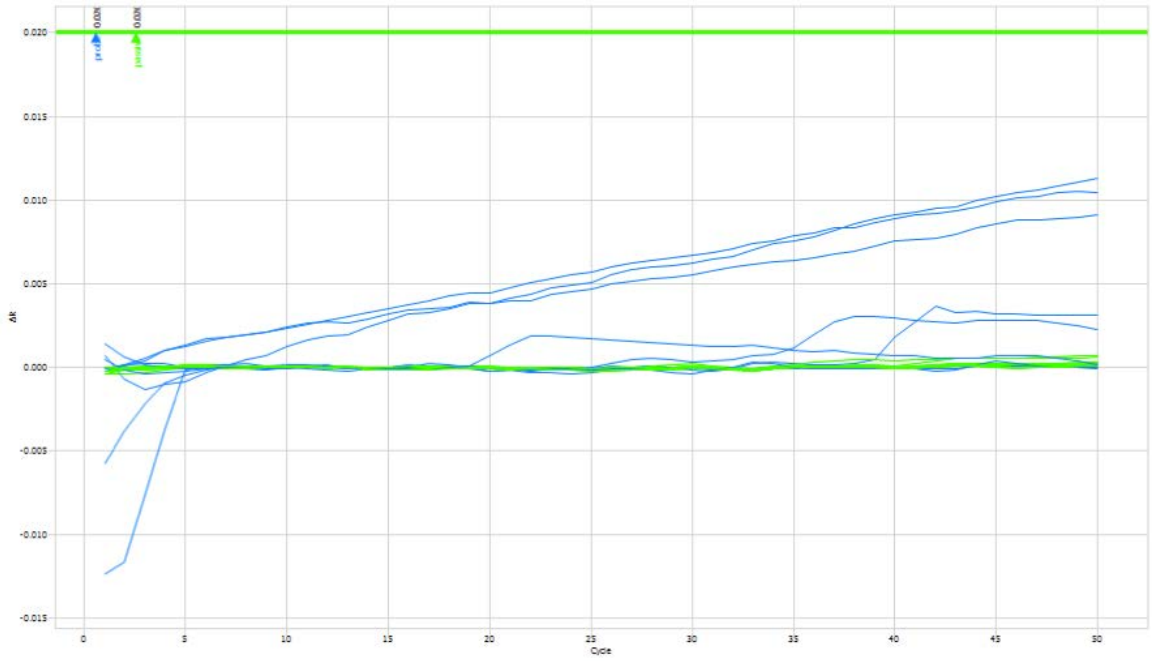
A. TaqMan Assays



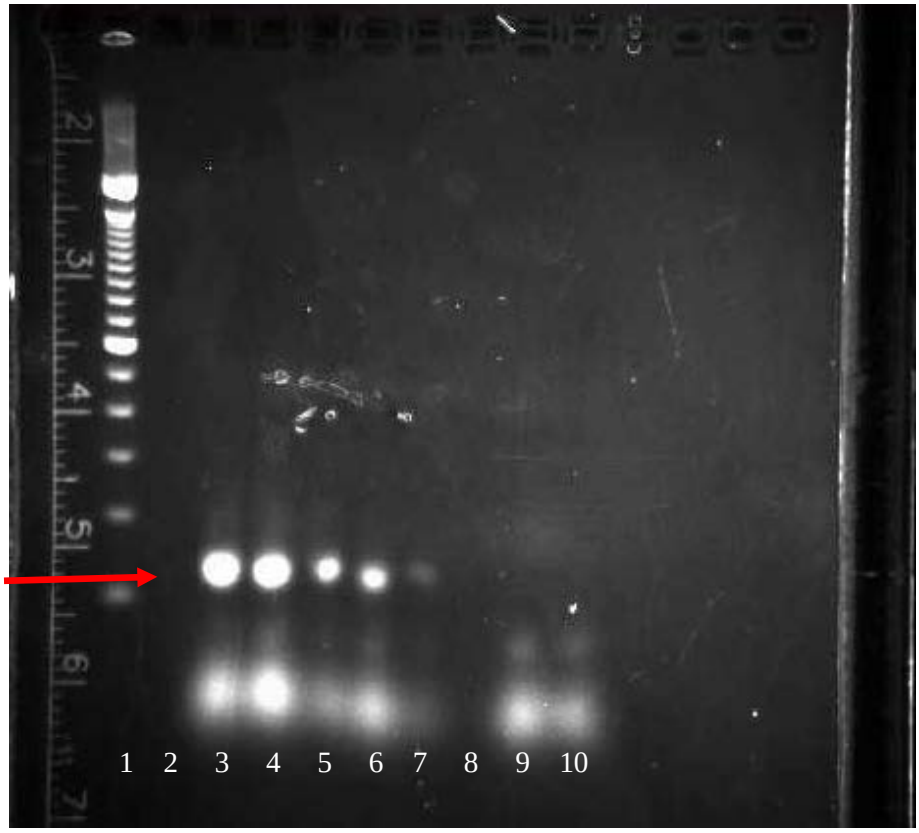
Fifth TaqMan assay amplification plot for chytrid standard DNA showing only background noise. Change in fluorescence is plotted against the cycle number.



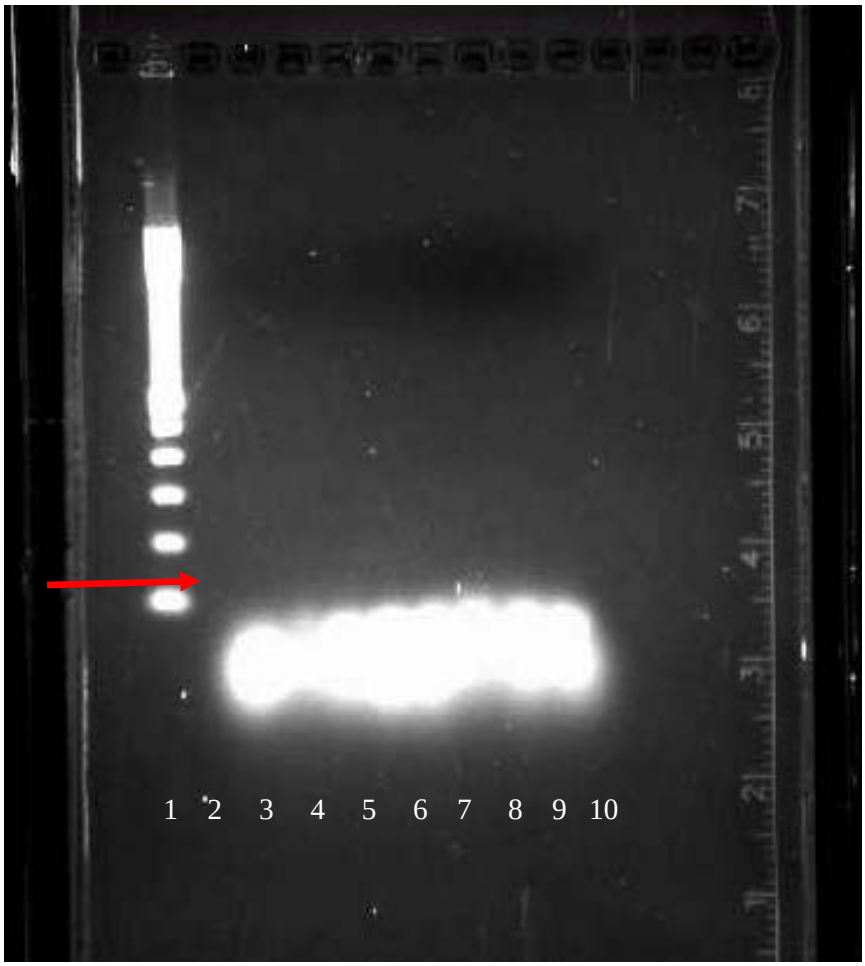
TaqMan assay amplification plot for chytrid standard DNA showing only background noise. Change in fluorescence is plotted against the cycle number.



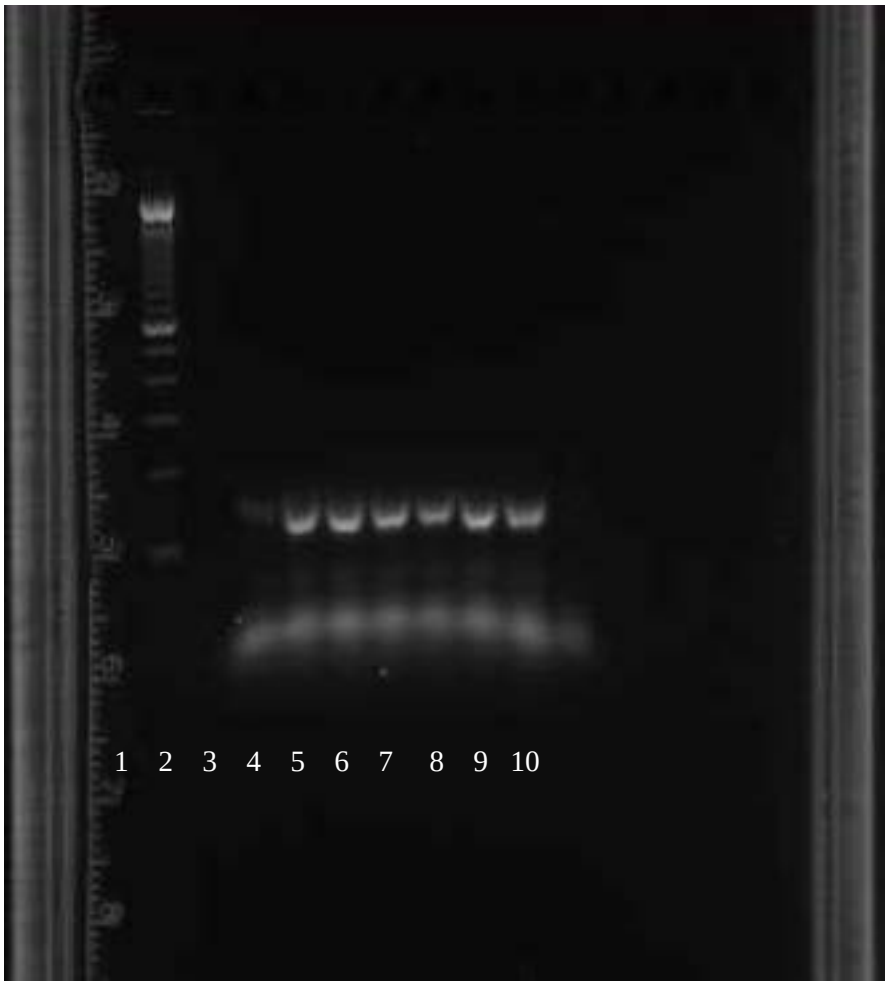
Typical TaqMan assay amplification plot for chytrid standard DNA showing only background noise. Change in fluorescence is plotted against the cycle number.



Agarose gel of real time PCR products from a TaqMan assay with an annealing temperature of 62^{O}C. Lane 1 contains a molecular weight marker and lanes 3 through 10 contain DNA concentrations of 420 000, 42 000, 4 200, 420, 42, 4.2, .42, and 0 molecules/reaction respectively. Arrow indicates expected location of target DNA.

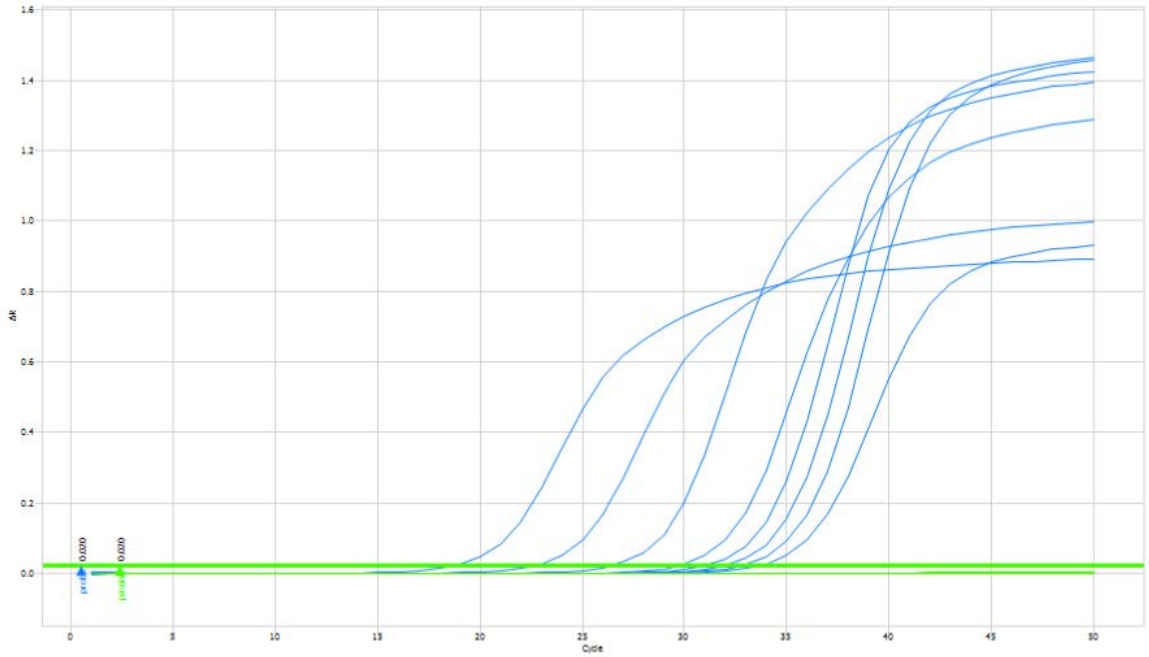


Agarose gel of real time PCR products from a TaqMan assay with an annealing temperature of 58^{O}C. Lane 1 contains a molecular weight marker and lanes 3 through 10 contain DNA concentrations of 420 000, 42 000, 4 200, 420, 42, 4.2, .42, and 0 molecules/reaction respectively. Arrow indicates expected location of target DNA.

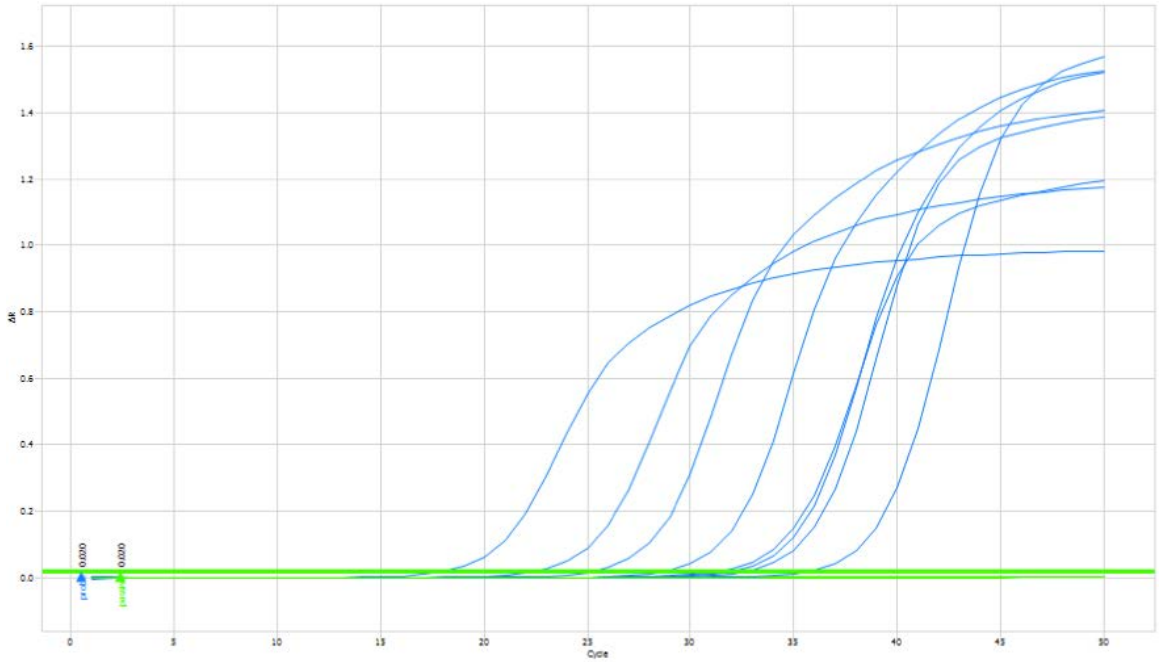


Agarose gel of real time PCR products from a TaqMan assay with an annealing temperature of 60^{O}C. Lane 1 contains a molecular weight marker and lanes 3 through 10 contain DNA concentrations of 420 000 molecules/reaction and probe volumes between 0.75 and 2.5 μL/reaction.

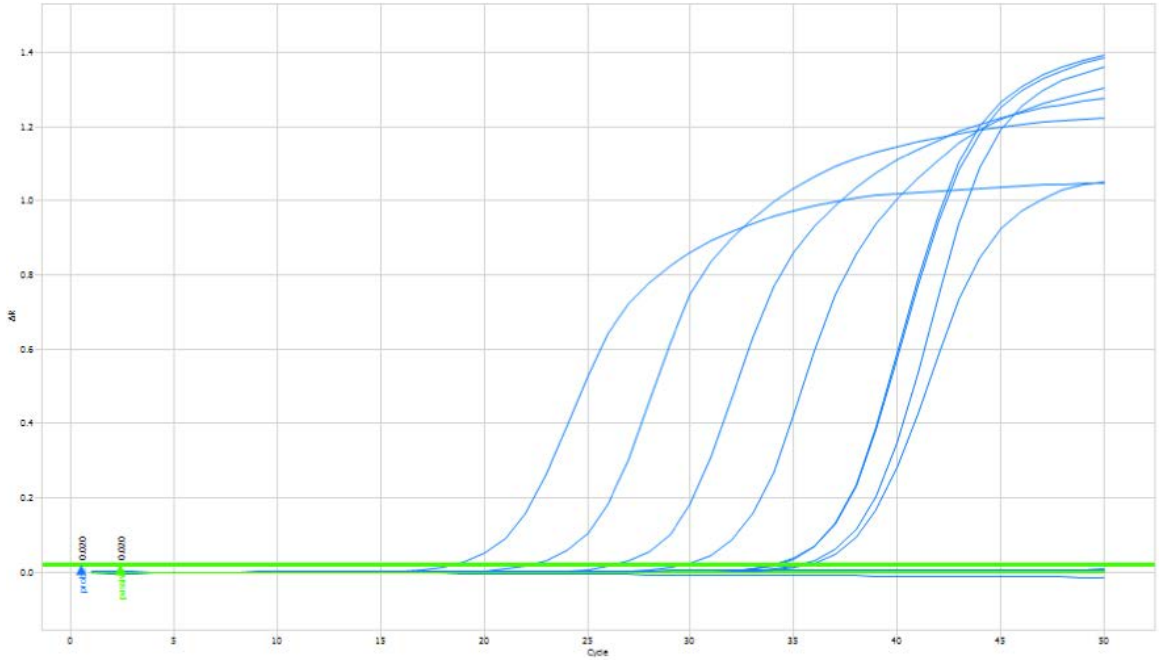
B. SYBR Green Assays



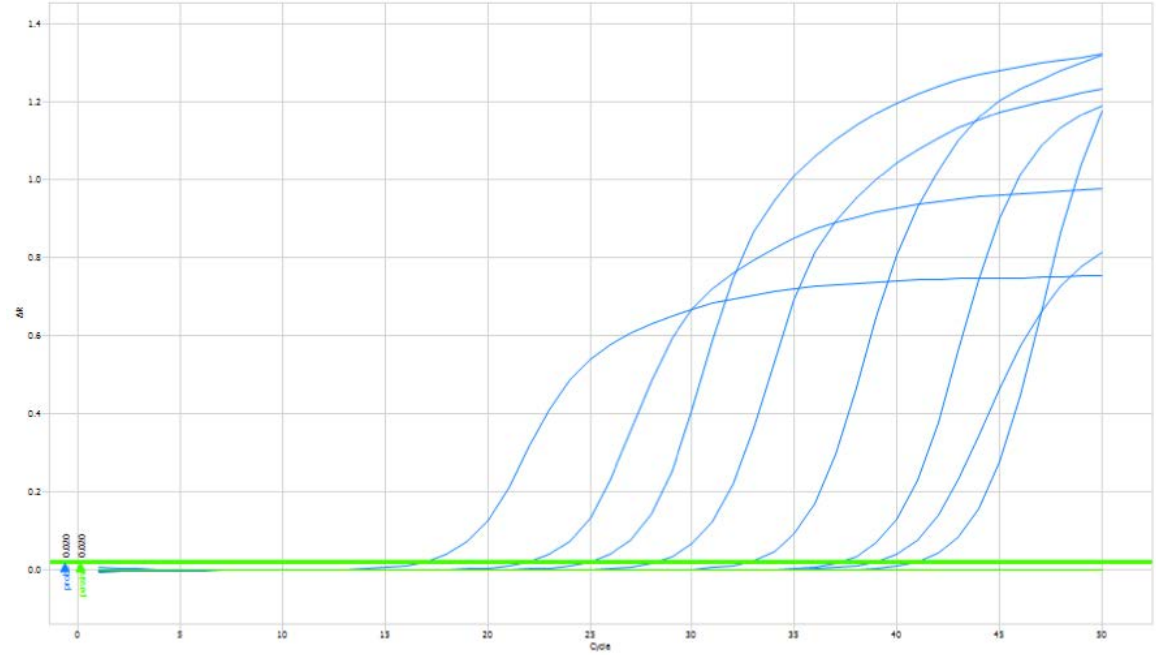
Amplification plot produced from the second real time PCR SYBR green assay using standard DNA and a cycling temperature of 60^{O}C.



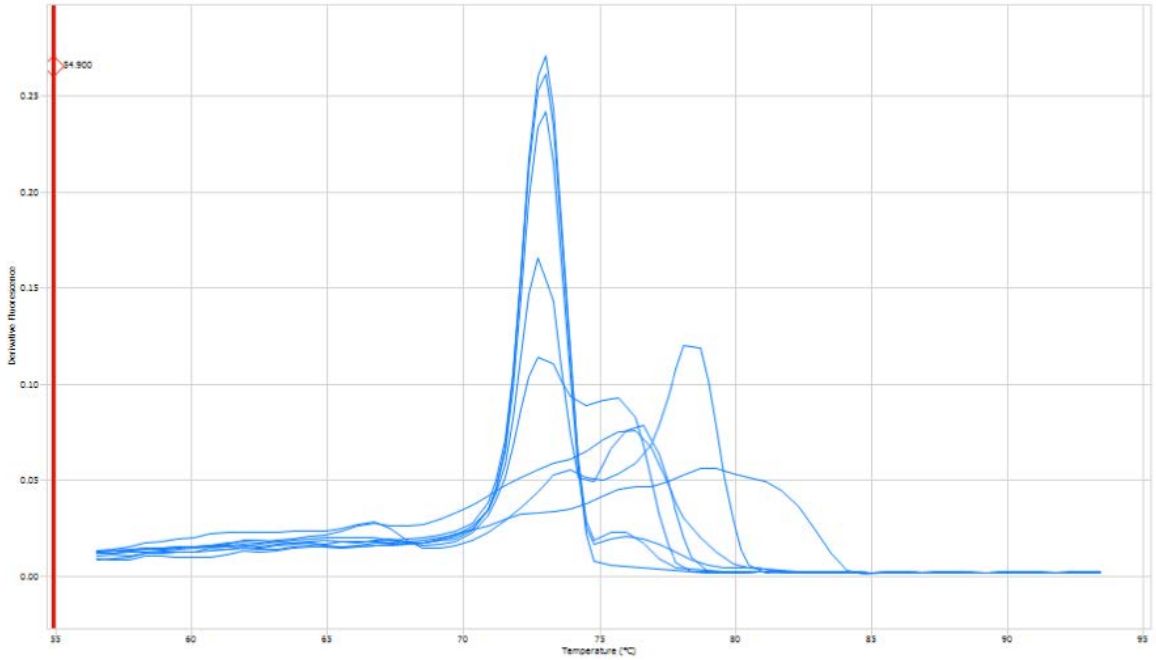
Amplification plot produced from the third real time PCR SYBR green assay using standard DNA and a cycling temperature of 60^{O}C.



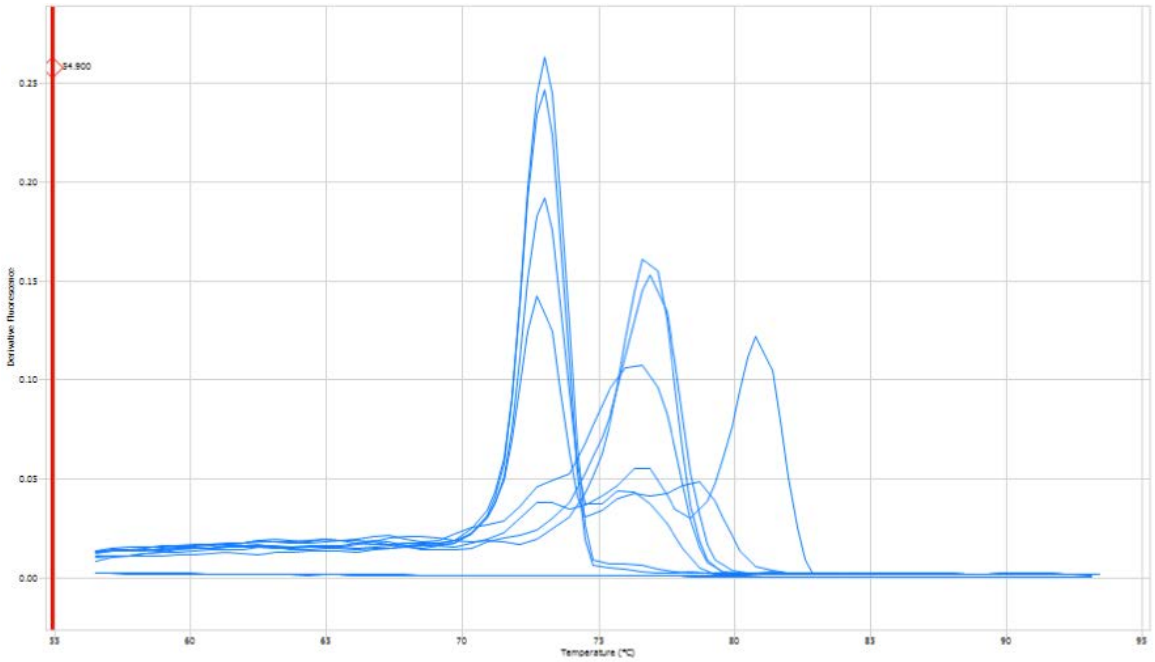
Amplification plot produced from the fourth real time PCR SYBR green assay using standard DNA and a cycling temperature of 60^{O}C.



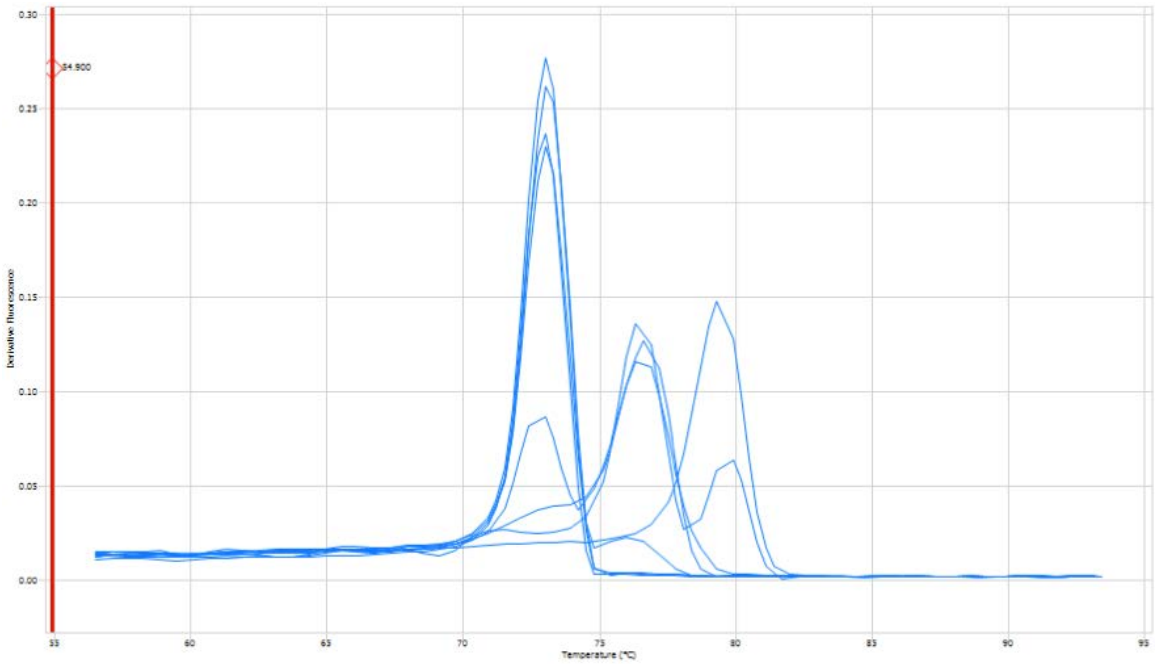
Amplification plot produced from the fifth real time PCR SYBR green assay using standard DNA and a cycling temperature of 60^{O}C.



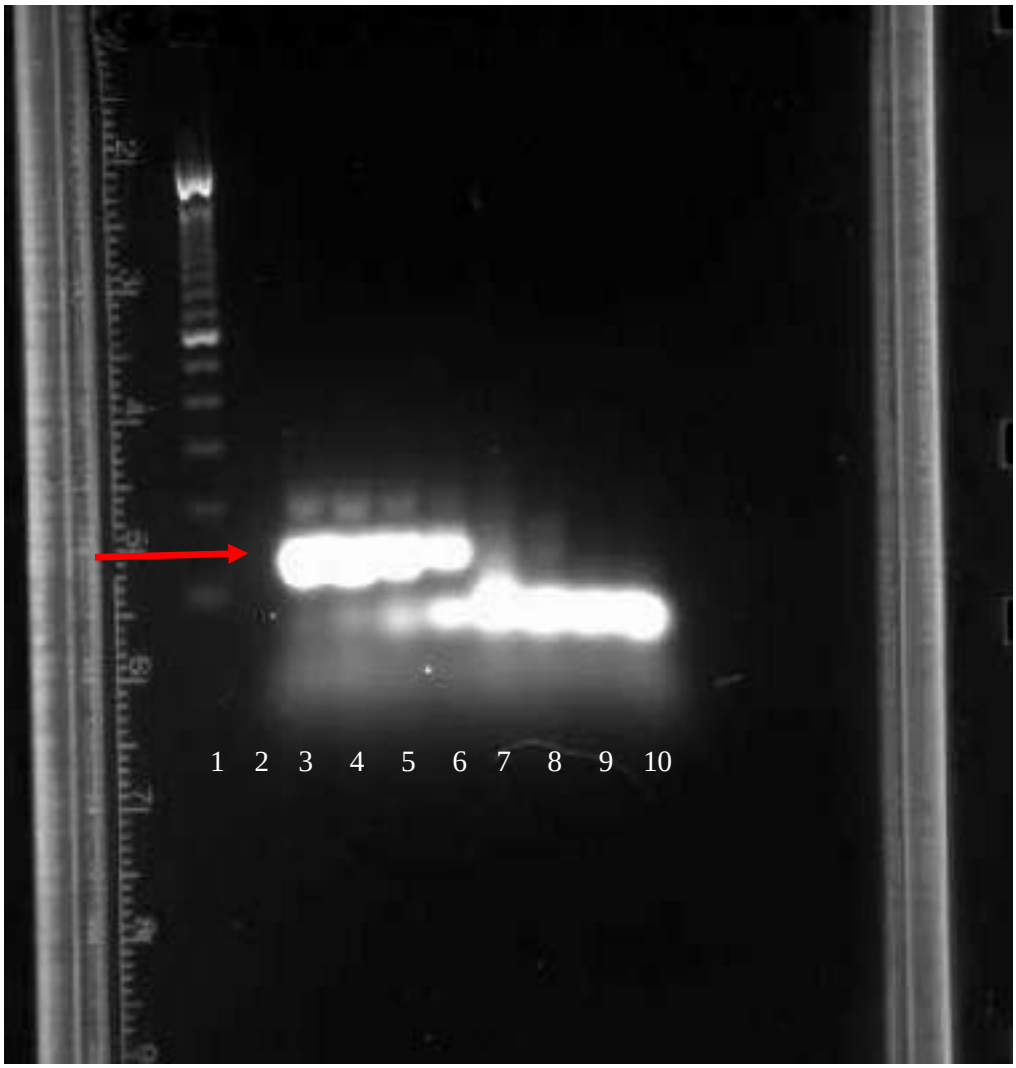
Melt curve analysis of the third real time PCR trial using the SYBR green buffer run at a cycling temperature of 60^{O}C.



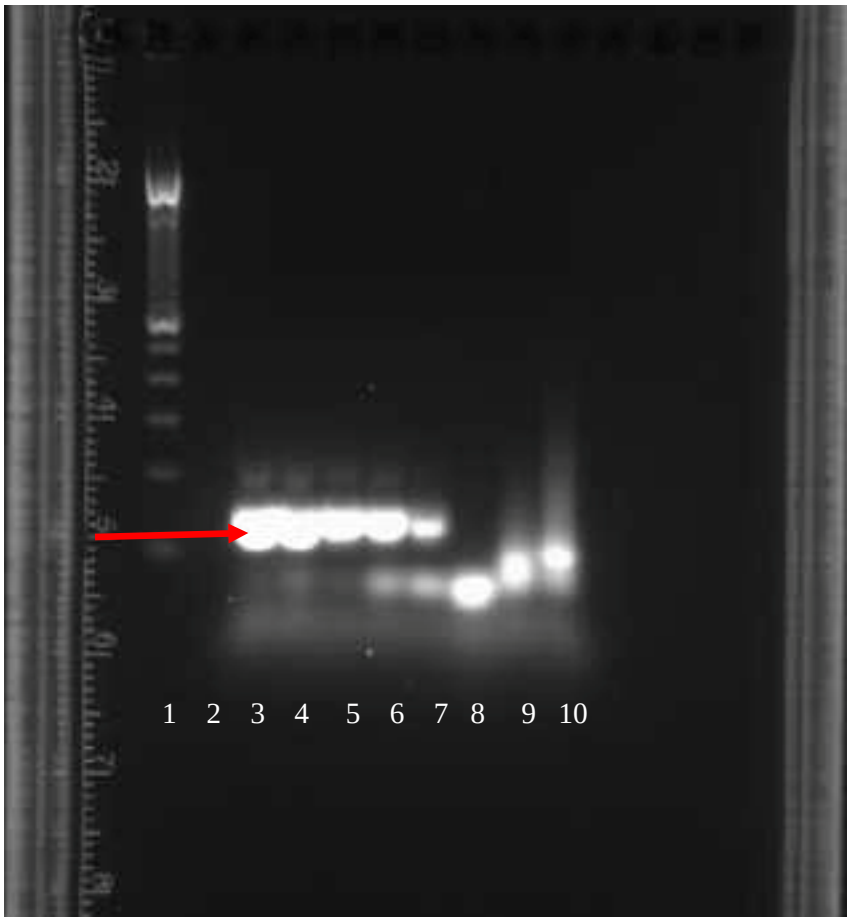
Melt curve analysis of the fourth real time PCR trial using the SYBR green buffer run at a cycling temperature of 60^{O}C.



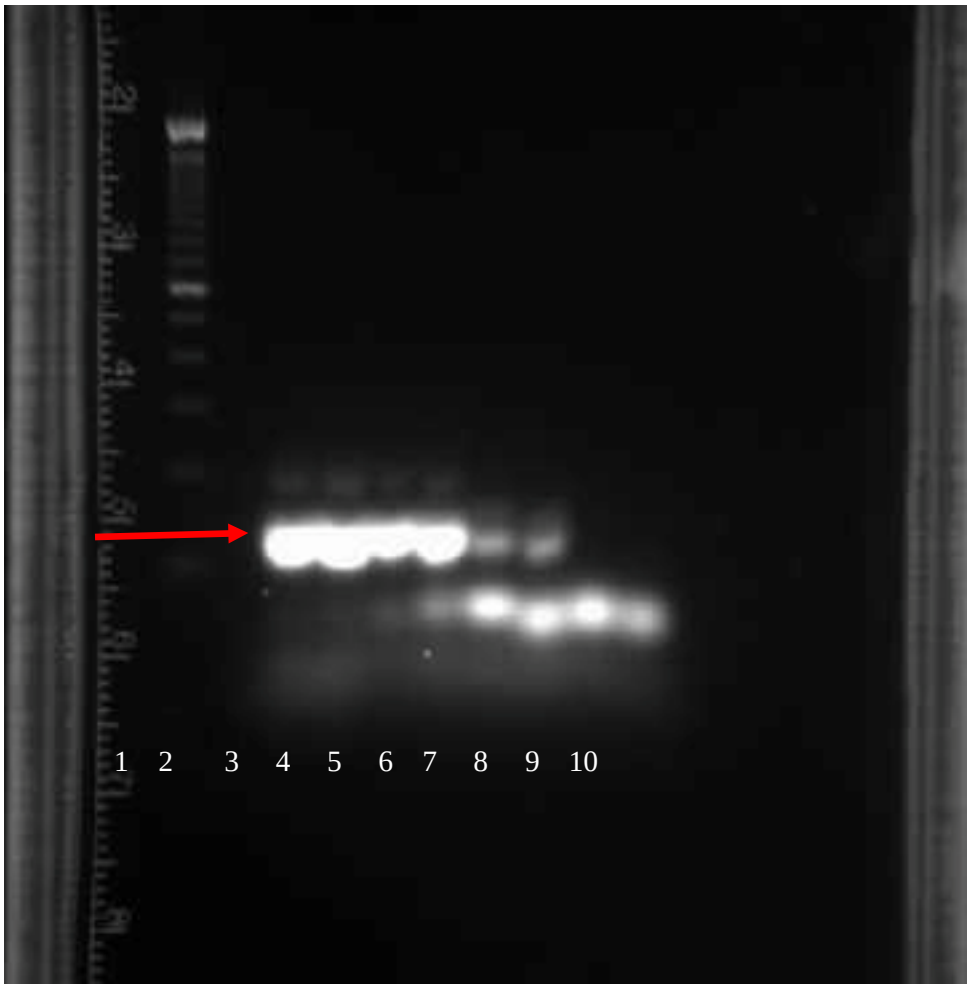
Melt curve analysis of the fifth real time PCR trial using the SYBR green buffer run at a cycling temperature of 60^{O}C.



Agarose gel of real time PCR products from the second SYBR Green assay.Lane 1 contains a molecular weight marker and lanes 3 through 10 contain DNA concentrations of 420 000, 42 000, 4 200, 420, 42, 4.2, .42, and 0 molecules/reaction respectively. Arrow indicates expected location of target DNA.



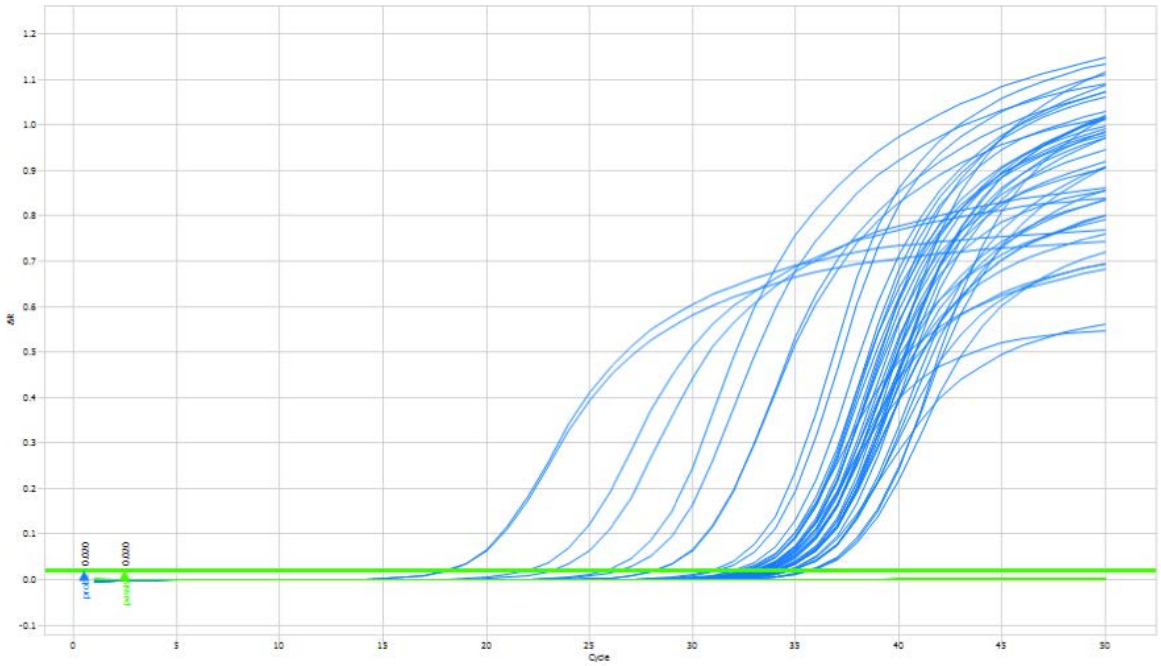
Agarose gel of real time PCR products from the third SYBR Green assay.Lane 1 contains a molecular weight marker and lanes 3 through 10 contain DNA concentrations of 420 000, 42 000, 4 200, 420, 42, 4.2, .42, and 0 molecules/reaction respectively. Arrow indicates expected location of target DNA.



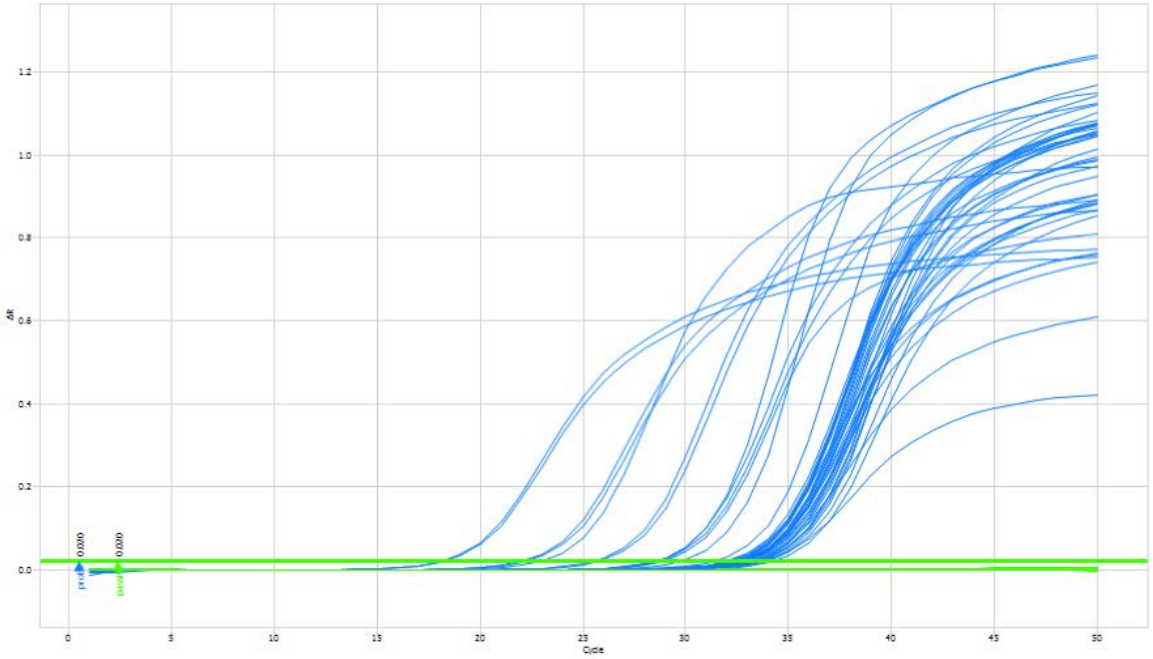
Agarose gel of real time PCR products from the fourth SYBR Green assay.Lane 1 contains a molecular weight marker and lanes 3 through 10 contain DNA concentrations of 420 000, 42 000, 4 200, 420, 42, 4.2, .42, and 0 molecules/reaction respectively. Arrow indicates expected location of target DNA.

Agarose gel of real time PCR products from the fifth SYBR Green assay.Lanes 1 and 13 contain a molecular weight marker and lanes 3 through 10 contain DNA concentrations of 420 000, 42 000, 4 200, 420, 42, 4.2, .42, and 0 molecules/reaction respectively. Arrow indicates expected location of target DNA.

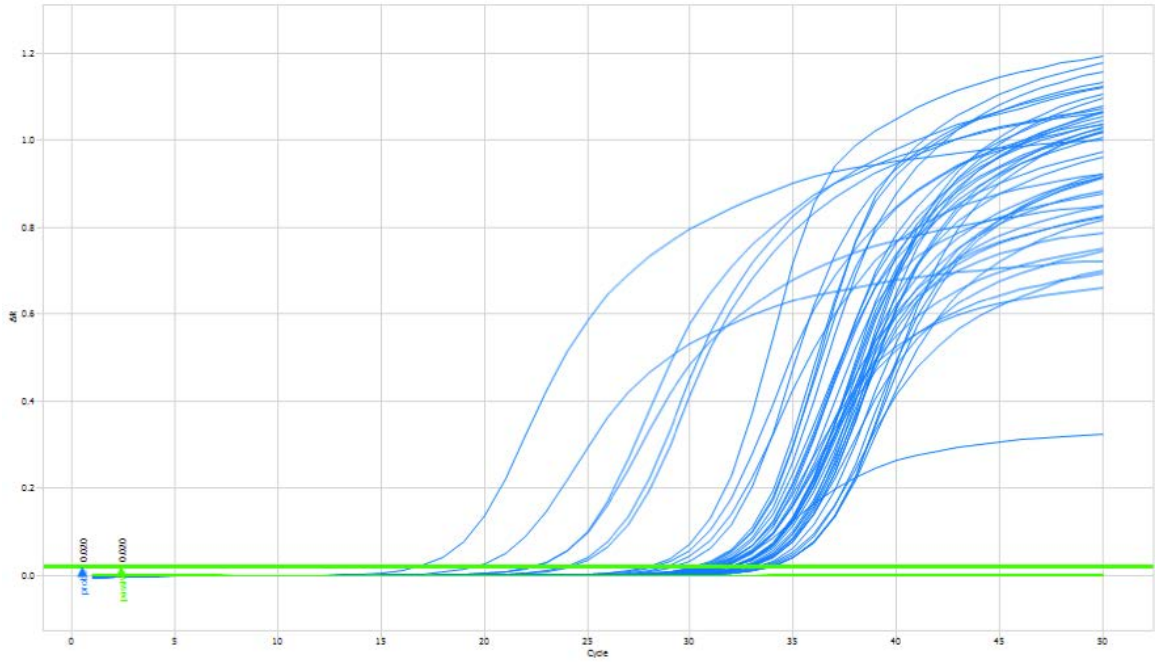
C. Real Time PCR analysis of environmental samples



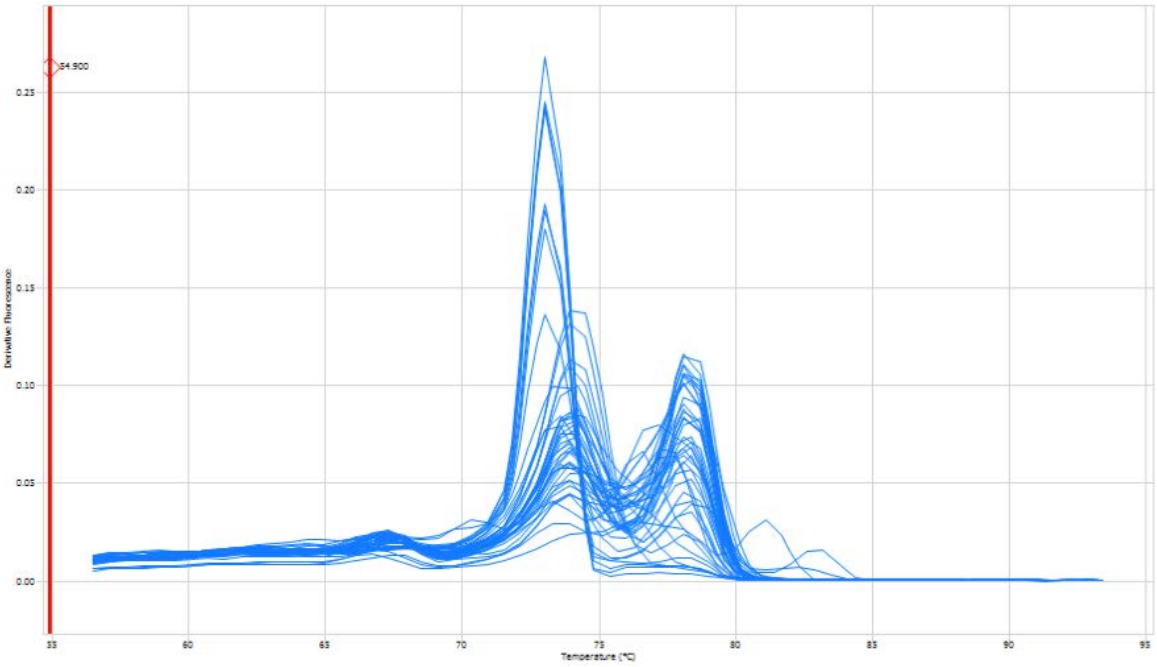
Amplification plot produced from a real time PCR SYBR green assay using standard DNA and 10 environmental samples.



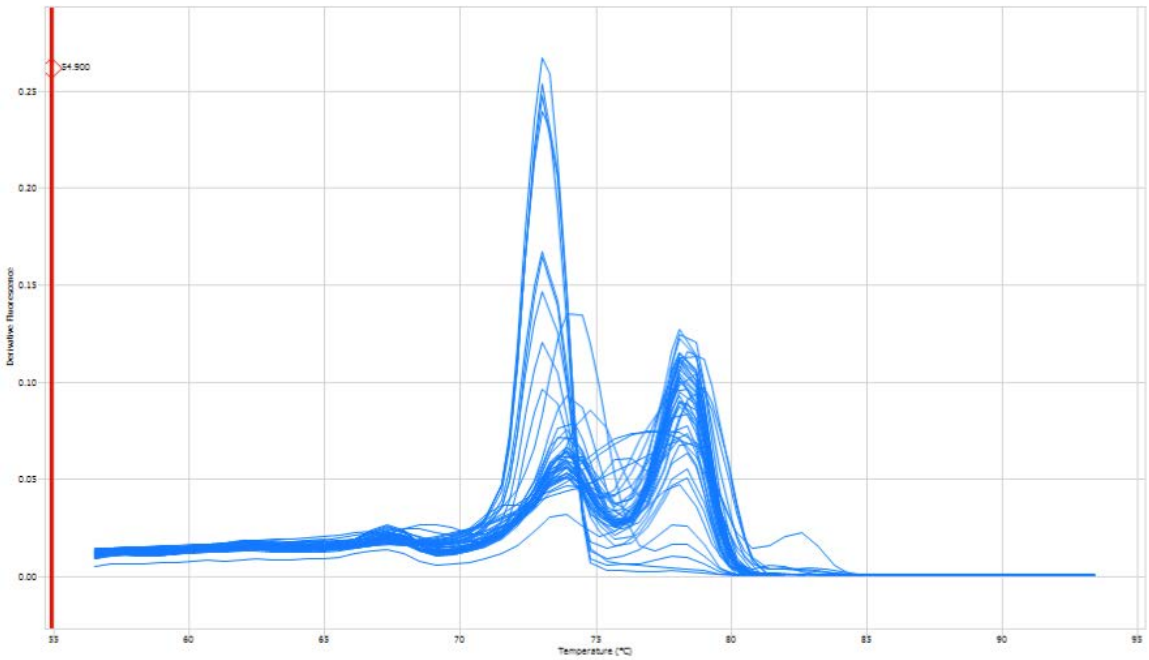
Amplification plot produced from a real time PCR SYBR green assay using standard DNA and 10 environmental samples.



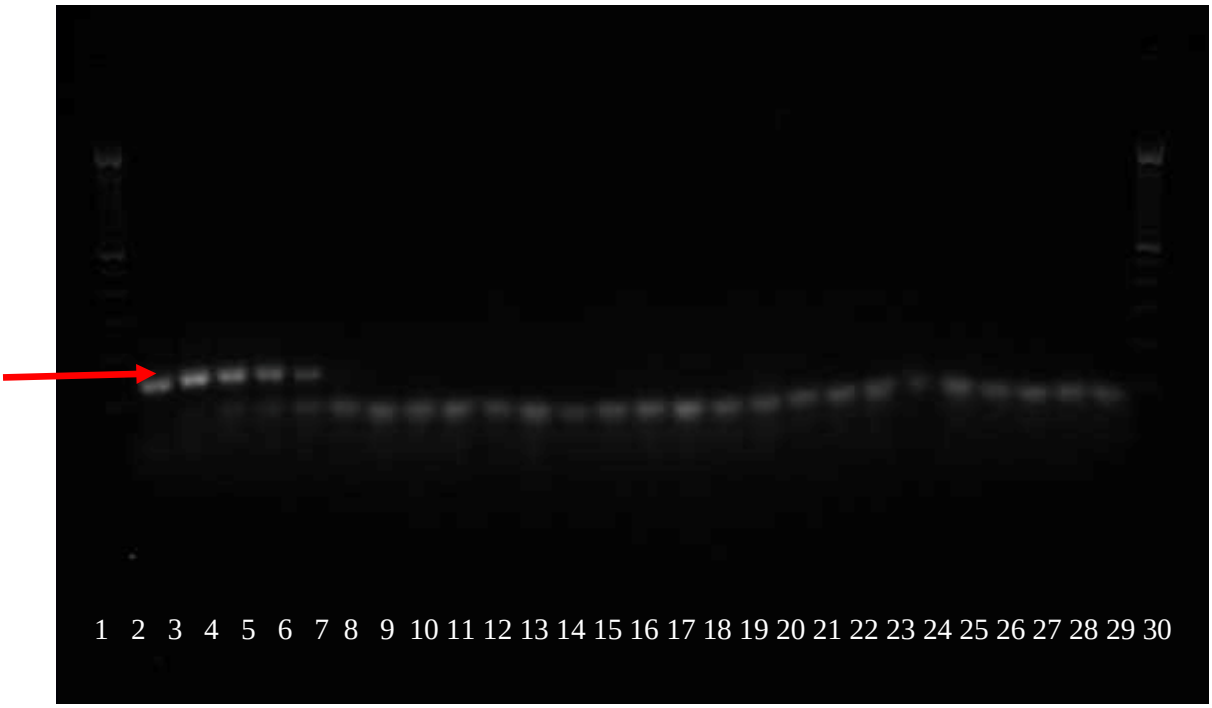
Amplification plot produced from a real time PCR SYBR green assay using standard DNA and 10 environmental samples.



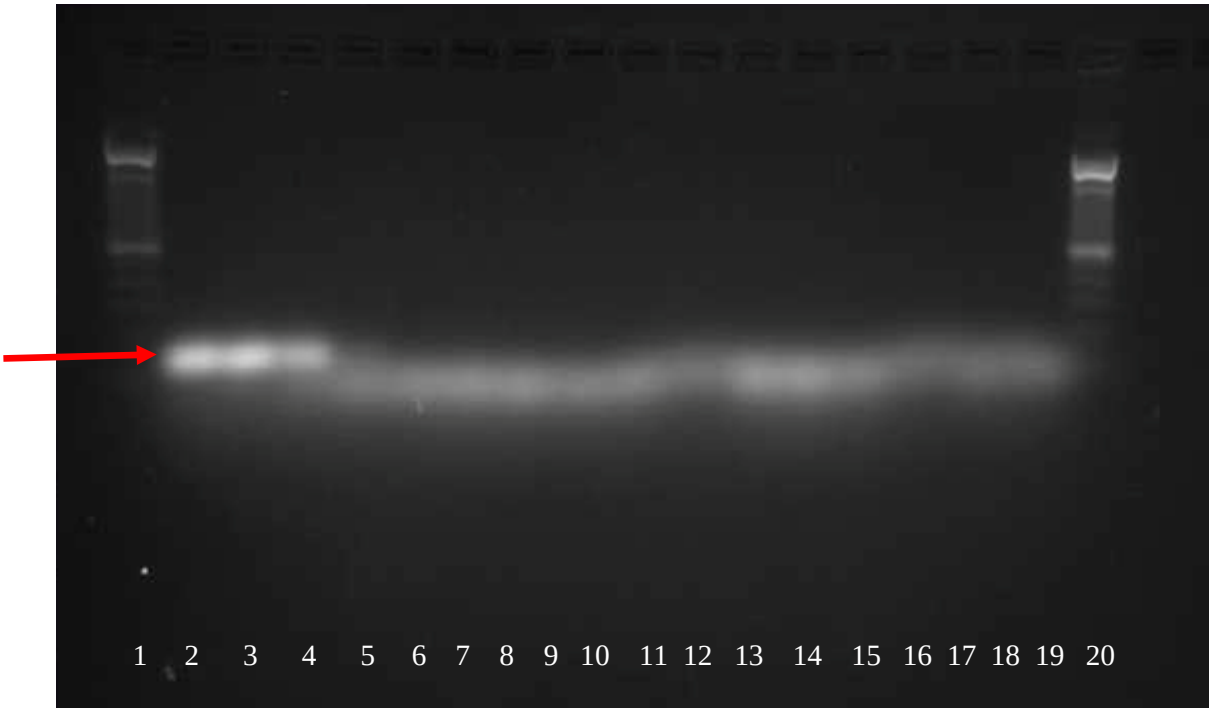
Melt curve analysis of a real time PCR trial using standard DNA and 10 environmental samples.



Melt curve analysis of a real time PCR trial using standard DNA and 10 environmental samples.



Gel electrophoresis produced using real time PCR products. Lanes 1 and 30 contain a molecular weight marker, lanes 2-9 contain DNA concentrations of 420 000, 42 000, 4 200, 420, 42, 4.2, .42, and 0 molecules/reaction respectively and lanes 10-29 contain environmental samples. Arrow indicates location of target DNA (146 bp).



Gel electrophoresis produced using real time PCR products. Lanes 1 and 20 contain a molecular weight marker, lanes 2-9 contain DNA concentrations of 420 000, 42 000, 4 200, 420, 42, 4.2, 0.42, and 0 molecules/reaction respectively and lanes 10-19 contain environmental samples. Arrow indicates location of target DNA (146 bp).

D. DNA Concentration on Environmental Swabs

Sample ID	DNA Concentration (ng/mL)
DP 29/5 (1)	1.59
S1 3/6 (1)	<0.5
PH 25/5 (8)	20.9
DP 29/5 (3)	7.18
PH 29/5 (1)	<0.5
DP 30/5 (2)	18
PH 5/6 (1)	<0.5
DP 30/5 (1)	12.4
DP 4/6 (2)	25
PH 4/6 (1)	11.9
LPH 20/6 (1)	4.49
DP 29/5 (7)	21.9
PLP 4/6 (1)	<0.5
DP 29/5 (5)	<0.5
PH 20/6 (1)	19.4
PLP 5/6 (1)	7.74
M 5/6 (1)	1.56
PH 5/6 (2)	<0.5
C 4/6 (1)	<0.5
S 11/6 (1)	34.8
E2 16/7 (1)	<0.5
E2 16/7 (2)	6.96
PH 20/6 (2)	11.8

PH 11/6 (1)	10.4
PH 5/6 (3)	8.63